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Rodent Leukemia: Recent Biological Studies. *A Review**

ARTHUR KIRSCHBAUM†

(Departments of Anatomy, University of Minnesota Medical School, Minneapolis 14, Minn., and of the College of Medicine, University of Illinois, Chicago 12, Ill.)

Understanding of the factors influencing the spontaneous development and the transplantation of experimental neoplasms has been sought in studies of rat and mouse leukemia. Since leukemia is induced readily in various strains of mice by either carcinogenic hydrocarbons, ionizing radiations, estrogens, or combinations of these agents (31, 66), the disease has been the subject of recent studies on experimental carcinogenesis. Because of their sensitivity to so-called "therapeutic" chemical agents, the leukemias of mice are being used extensively in screening programs (16) and for the study of cellular refractoriness to agents such as the folic acid antagonists (19, 72).

Morphological types.—The usual type of systemic leukemia is lymphoid. Myeloid leukemia, often chlorotic, appears relatively infrequently (67). Spontaneous mediastinal (thymic) lymphosarcoma occurs primarily in young mice (49, 67). Unusual distribution of leukemic infiltrations may characterize the disease in hybrids, the patterns of disease being more uniform in the pure strain (49). In "high-leukemia" strains the incidence may be as high as 90 per cent, the majority of cases appearing by 1 year of age. Leukemia occurs spontaneously in old animals, beyond 500 days of age, of strains such as the CBA and Bagg albino which are considered "low-leukemia." If careful autopsies were done routinely on populations given good care, it would probably be found that in old mice of even the "leukemia-resistant" strains the disease develops in as many as 5 per cent.[1] It can be very difficult to decide in old mice upon gross examination whether the large Malpighian corpuscles of the spleen are hyperplastic or actually representative of leukemic neoplasia.

The interactions of genetic and extrinsic factors determine the time of appearance and the type of lymphomatous and myelomatous disease. In the Ak stock and in certain hybrid crosses with a low-leukemia strain (F_1 and backcross to the Ak), neoplastic disease of the hemopoietic system appears early, and mediastinal lymphosarcoma is common. In backcrosses to the low leukemia stock, leukemia develops late, and localized mediastinal disease occurs rarely (49). In strains which develop spontaneous leukemia or lymphosarcoma only occasionally (as well as in certain ones with a high incidence), ionizing radiations, carcinogenic hydrocarbons, or estrogenic hormones may induce lymphomatous disease (65). Susceptibility to each of these agents is determined by genetic factors which are not identical with those determining susceptibility to spontaneous disease.

Rare types of rodent neoplasms of hemopoietic tissue are the hepatic histiocytoma (42), which on transplantation may express itself as a leukemia or sarcoma of the stromal reticulo-endothelial tissue, plasma-cell leukemia (100), and mast-cell tumors (24). Reticular cells may predominate in some rat and mouse lymphomas, the term "reticulum-cell sarcoma" having been applied. Al-

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† Present address: College of Medicine, University of Illinois, Chicago 12, Ill.

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[1] A. Kirschbaum, unpublished data.

though atypical giant cells and fibrosis may appear in the infiltrations, the "Hodgkins" adjective is probably not appropriate.

Cytology.—The "immature" leukemic cells, as seen in blood and marrow smears, and imprints of the infiltrated organs, closely resemble the undifferentiated human leukemic cells (67). Distinctive cytological characteristics such as granulation may distinguish transfer lines.

An increase in the number of mitochondria occurred during potentiation of malignancy within one transfer line, whereas a second transfer line whose infiltrative capacity remained unaltered exhibited no similar cytologic modification (86).

Chromosomes of mouse leukemic lymphocytes are larger than those of homologous normal cells of the adult. Their size is greater in the more malignant cells of transplanted leukemia as compared to lymphocytes of spontaneous leukemia. Lymphocytes of the embryo also possess large chromosomes. The difference in the size of the chromosomes of normal and leukemic cells is due to amounts of pepsin-digestible protein (11). Sex hormones influence the size of the chromosomes in mouse leukemic cells (12).

The whole "nuclear fraction" of leukemic spleens is extremely rich in RNA, as compared to the similar fraction from normal spleens. This may be accounted for by the fact that the nuclei of leukemic spleens contain nucleoli which are not present in normal mature lymphocytes (95).

No cytologic alteration could be correlated with the development of refractoriness in a transfer line of leukemia to the inhibiting action of folic acid antagonists (19, 72).

Although folic acid antagonists inhibit the development of certain transplanted leukemias, there seems to be no "radiomimetic" effect, as seen in the action of nitrogen mustards (7), colchicine (77), and alpha- and beta-peltatin (44), where pyknosis and nuclear fragmentation occur. Massive doses (several times the LD_{50}) of nitrogen mustards may kill leukemic cells, so that transplantation is not possible from treated animals. On the other hand, treatment of leukemic mice with extremely large doses of the folic acid antagonists does not alter transplantability (14).

An increased percentage of mature cells appeared in the blood, and the total white blood cell count dropped to normal levels following the administration of urethan to mice with a transplanted myeloid leukemia. Similarly, there was a "shift to the right" in the bone marrow. Correlated with the accelerated maturation of leukemic cells there was a decreased number of mitotic figures (64).

Cellular morphology may indicate fundamental differences in leukemic cells. Myeloid leukemic cells of the F strain are usually sensitive to the action of urethan and potassium arsenite, although resistant to folic acid antagonists. The reverse is true for lymphoid leukemic cells (63). Within leukemias of one cell type, tremendous variations in response may occur.

Leukemogenic Factors

Genetic.—The most carefully controlled work reveals that the gene may definitely influence the development of mouse leukemia (81). Females of the low-leukemia StoLi strain were mated with a single C58 (high-leukemia) male, with 7 F_1 sons and with 50 backcross (to StoLi) grandsons. All mice were nursed by Bagg albino females. The incidence of leukemia in the offspring of StoLi females mated with backcross fathers varied from 0 to 42.8 per cent, indicating the genetic diversity of the backcross males and suggesting a segregation of the genes influencing leukemia.

The length of life of the offspring was a complicating factor in evaluating results. Length of life was affected by the genetic pattern of the backcross fathers, by a nursing influence from the females, and by sex itself, females living longer than males. Increasing the parturition age of the mother delayed the appearance of leukemia without influencing the other causes of death, so that potential leukemics died of other causes.

When high-leukemia C58 mice were originally reciprocally crossed with low-leukemia StoLi mice, a maternal influence analagous to that observed for mammary cancer was suggested (82), since the incidence of leukemia in genetically similar F_1 hybrids was higher if the female parent was of the high-leukemia C58 strain. It now appears that the maternal influence responsible for this difference consists of a definite resistance to leukemia contributed by the low-leukemia strain mothers (83). This may be supplied before birth or by nursing alone. It becomes an increasingly potent influence with advancing age. When this resistance is absent, the leukemic heredity of the C58 strain, whether introduced by the male or female, shows dominance. It appears that this "resistance factor" contributed by StoLi mothers extends to other causes of death.

Nongenetic factors influence the development of leukemia and complicate the situation sufficiently so that it is probably premature to conclude from one series of crosses that a single dominant gene controls the development of leukemia, and that leukemic cells develop as a result of somatic mutation in cells with this labile gene (49).

Leukemia has been generally observed to appear later in life in F_1 hybrids and in backcrosses to the low-leukemia strain than in the pure strain or its backcrosses (49, 62). The time of appearance of leukemia in the heterogeneous F_2 population is variable. Early development of the disease tends to be manifested as thymic lymphosarcoma.

The genes determining susceptibility to the development of "spontaneous" leukemia are not identical with those involved in the reactions to leukemogenic agents such as ionizing radiations, carcinogenic hydrocarbons, and estrogenic hormones. Strains with a relatively low incidence of spontaneous leukemia (e.g., CBA) may respond readily to a specific leukemogenic agent (e.g., estrogen), but not to another such as methylcholanthrene (65).

Viral.—Although in F_1 hybrid reciprocal crosses between certain high- and low-leukemia strains the incidence of leukemia is higher and the age of onset earlier if the female parent is of the high-leukemia strain, foster nursing experiments do not support the suggestion that a "leukemia agent" is present in the milk, as in the case of mouse mammary cancer (68). Although a virus may be associated with certain transfer lines of leukemic cells (116), it is considered to be a contaminant in so far as the etiology of leukemia is concerned.

When very young mice of the low-leukemia C3H strain were inoculated with a noncellular extract of either leukemic tissue, or of embryonic cells from the high-leukemia Ak strain, leukemia appeared "spontaneously" (46). Susceptibility to the agent was expressed only by mice inoculated at less than 12 hours of age. It is suggested that mouse leukemia may be transmitted by an agent analogous to that of chicken leukosis. Confirmation of these findings is required to establish mouse leukemia as a viral disease. Several investigators have suggested the possibility that mouse and rat leukemia may be transmitted by cell-free materials (112). Freezing followed by lyophilization at low temperatures inactivates the capacity of leukemic tissue to transmit the disease to animals susceptible to cellular transmission (34).

Hormonal.—Estrogenic hormones, when given in appropriate doses, are leukemogenic for certain strains of mice (38) and their hybrids (36, 37). The resulting type of disease is usually thymic lymphosarcoma. The leukemogenic effects of x-rays or methylcholanthrene may be potentiated by exogenous estrogen (66). In certain high-leukemia strains (88), but not in others (81), the incidence of spontaneous leukemia is higher in females than males.

Androgenic hormones are anti-leukemogenic in mice under certain conditions. The leukemogenic action of estrogens is nullified by the simultaneous administration of appropriate amounts of androgen (38). The incidence of radiation-induced lymphomas is likewise reduced by administering testosterone propionate (35, 58). Injections of androgenic hormone reduced the incidence of leukemia in females to that found in males of the RIL strain (88). Although orchidectomy increased the incidence of leukemia in RIL males, the incidence of the radiation-induced disease of C57 black mice was not significantly altered by gonadectomy (58).

Adrenalectomy increased the incidence of spontaneous leukemia in C58 mice (73) and of radiation-induced thymoma in C57 blacks (59). Administration of cortisone decreased the percentage of induced lymphomas in the latter strain (59). Adrenalectomy is said to increase susceptibility to transplantation of rat lymphosarcoma (115), whereas cortical hormone exerts inhibitory effects (91).[2]

Lymphosarcomas originating in the lung appeared in six of fifteen rats treated with growth hormone, whereas none appeared in controls. In all the injected animals the peribronchial lymphoid tissue was hyperplastic, and it was from this site that the lymphosarcomas developed (87).

Carcinogenic hydrocarbons.—The susceptibility of certain strains of mice to the induction of leukemia may depend primarily upon the response of the thymus. When small amounts of carcinogenic hydrocarbons were implanted directly into the various organs, local lymphoid tumor development appeared in the thymus but not in the other lymphoid organs (98). The most potent of the carcinogens upon subcutaneous injection was 9,10-dimethyl-1,2-benzanthracene. Since 3,4-benzpyrene, 1,2,5,6-dibenzanthracene, and 20-methylcholanthrene are effective when injected directly into the thymus, it appears that rapidity of absorption may determine leukemogenic potency. The site of injection may be decisive in determining leukemogenic effectiveness (97, 99).

Removal of the thymus reduced the incidence of carcinogen-induced leukemia from 69.7 to 22.0 per cent (75). Removal of the spleen or transplantation of one or two thymuses into intact DBA mice did not alter the incidence of carcinogen-induced leukemia. If, however, thymectomy was followed by autoplastic grafting, the incidence of induced leukemia was 69.1 per cent, indicating that the presence of thymic tissue is necessary for the development of leukemia in a high percentage of

[2] J. Stasney, K. E. Paschkis, and A. Cantarow, personal communication, 1951.

mice of this strain. Although, in contrast to Ak mice (85), the thymus is apparently not ordinarily the primary locus for the development of spontaneous leukemia in C58 mice, its removal does reduce the incidence (76). Grafted thymuses did not appear to serve as the focus for lymphoma development. Absence of the thymus did not change the incidence of other types of induced tumors.

Although *p*-dimethylaminoazobenzene is usually considered carcinogenic for the liver alone, 5 of 28 rats receiving splenic implants developed lymphoblastic lymphosarcomas (78). No similar tumors appeared in controls, nor were neoplasms of this type induced by oral administration.

Following gastric instillation of methylcholanthrene into 59 Wistar rats, 6 developed lymphatic and 2 myelogenous leukemia (104). Leukemia appeared in young rats suckled by mothers which received methylcholanthrene by stomach tube. Labeled 20-methylcholanthrene was recovered from the milk of the stomachs of the offspring, indicating that carcinogenic agents may be transferred to the offspring by way of the breast milk (105).

Radiation.—Lymphosarcoma and/or leukemia may be induced by the whole-body radiation of mice. The thymus of C57 blacks seems to be primarily susceptible to this action of x-rays, disseminated disease (leukemia) resulting from secondary infiltration of other organs (53). Radiation of the thymus alone, or of the whole body except the thymus, did not result in the induction of thymic tumors (55, 56). That a humoral factor operates is indicated, since shielding of an extremity decidedly reduced the incidence of induced thymomas (56). Normal nonirradiated tissue may inactivate a humoral factor produced by radiation.

If a strain of mice is susceptible to the induction of leukemia by x-radiation, the younger the animal is at the time of exposure to x-rays (54), the greater *its* susceptibility. Estrogen enhances (35, 66) and androgen decreases susceptibility (35, 58). Certain strains are more susceptible to the induction of leukemia than others; in one the thymus may be the target organ (53), whereas in others the locus seems frequently to be extrathymic.[1] Treatment with estrogenic hormone may render lymphoid tissue susceptible to the induction of neoplastic change by local radiation.[1] In support of the idea that a humoral factor resulting from radiation is the actual leukemogen is the observation that in estrogen-treated mice thymic lymphosarcoma may be induced by radiation of the entire body except the thymus.[1]

The incidence of lymphoma was proportional to the total dose of radiation in C57 black mice, whether fractionated or in one exposure (57). With fractionation treatment, if the intervals between radiation were 4–8 days, the incidence was greater than if treatments were given daily. If the intervals were 16 days the incidence was no greater than for daily treatments.

Nutritional.—Nutritional factors may influence the time of onset of either spontaneous or induced leukemia, or alter the growth of transplanted lymphoid tumors. Caloric restriction resulted in a later appearance and reduction of the total incidence of leukemia in mice of the Ak strain (101). The incidence of carcinogen-induced leukemia was remarkably reduced in DBA mice when the animals were fed a diet low in cystine (119). That the effect was not one resulting from nonspecific inhibition of growth is suggested by the observation that the restriction of other amino acids (lysine or tryptophan) decreased the body weight but not the incidence of leukemia.

Both pyridoxine and riboflavin deficiencies were associated with the inhibition of growth of certain transplanted lymphosarcomas (113, 114). Administration of a guanine analogue, 8-azaguanine (guanazolo), has been reported to inhibit the growth of transplanted leukemia (60, 71). It has been postulated that the effect is caused by interference with the utilization of guanine, which is essential for the growth of neoplastic cells (61). The inhibitory effect of folic acid analogues on transplanted leukemia has been attributed to interference with folic acid metabolism resulting in a diminished nucleic acid synthesis (107).

Transplanted Leukemia

Genetic factors control the transplantation of leukemic cells. Cells of specific lines proliferate only in mice of certain genetic constitution. Leukemic cells are usually 100 per cent transplantable into the mouse strain of origin and into F_1 hybrids between this and the foreign strain.

Transplantation patterns (susceptibility of hybrids of varied genetic constitution) of normal and malignant cells are different, an observation which has been interpreted to indicate that the genic composition of malignant cells differs from that of normal cells (32). The percentage of F_2 hybrid mice supporting the growth of leukemic cells indicates the number of genes involved in transplantation.

Malignant lymphocytes grow not only when transplanted into a suitable living animal medium, but also in tissue culture if a mesenchymal stromal tissue is present (23). The stromal tissue itself cannot be retransplanted into animals and grow as a tumor, which observation indicates that the lym-

phoblastic round cell is the essential malignant cell. In peritoneal ascitic fluid malignant lymphocytes proliferate, apparently increasing in number in the absence of peritoneal implants (41).

The rate of growth of transplanted mouse lymphosarcoma cells was not more rapid than that of normal cells of 12-day-old hosts (93); when the same lymphosarcoma was inoculated into 42-day-old mice, the animals with tumors gained more weight than littermates without tumors—the tumors accounting for weight gain (92).

Heterologous and cross-strain transplantation of mouse leukemia have been accomplished by x-irradiation of the foreign species or mouse strain prior to inoculation of leukemic cells (51). Attempts to transplant lymphomas into foreign species by inoculation into the anterior chamber of the eye have been unsuccessful (79). This lack of growth has been explained on bases other than nonmalignancy and/or absence of autonomy. Mouse lymphomas are malignant by all criteria other than growth in the anterior chamber of a foreign species. Failure to grow may be attributed to the lack of resistance of lymphoma cells to various types of trauma, the short life span of the cells, and the rapid development of immunity in the inoculated host. Mouse lymphomas are transplantable into the eyes of only homologous mouse strains.

When inoculated into a foreign strain, mouse leukemic cells may form a temporary growth which subsequently regresses. The hosts resist a second transplantation of the same line of leukemic cells, no "temporary" growth appearing. This capacity of certain leukemic cells to grow in foreign strains is said to be evidence of their high resistance to the action of antibodies. Inability of regressing leukemic cells to grow upon transplantation into an ordinarily susceptible host is attributed to adsorbed antibody. The serum of "immune" animals may inactivate leukemic cells *in vitro*, so that their ability to transmit the disease is lost (43). Similar *in vitro* inactivation of leukemic cells occurs when they are placed in contact with antiserum prepared by inoculating leukemic cells into a foreign species (118). Antigen is present in both normal and malignant cells, but to a greater extent in the latter (26). Cytoplasmic material is more highly antigenic than nuclear (4, 25). Nonspecific inactivating material of rabbit serum can be destroyed by heating at 56° C. (118). Antiserum against lymphosarcoma cells (produced in rabbits) inhibited the growth of a transplanted rat lymphosarcoma (94).

The temporary growth of leukemic cells in foreign strains of mice may be inhibited or stimulated by multiple prior injections of frozen, lyophilized tissue (109, 110). Stimulation or inhibition depends on both the source of tissue and the neoplasm transplanted. Animals may actually succumb as a result of invasion of cells which ordinarily grow only temporarily. Foster-nursing may render animals of otherwise resistant strains susceptible to the transmission of leukemia (69).

Immunity to mouse leukemic cells has been induced in mice by inoculation of normal tissue of a foreign strain or a sublethal dose of leukemic cells prior to injection of a lethal dose (84). The immunity produced in the latter manner was passively transferred by inoculation of spleen or liver of immune animals; that induced by the administration of normal tissue was not transferable (96). Although it was possible to immunize against transplanted leukemia, such immunity could not be induced against spontaneous leukemia nor the cells from a spontaneous case (80).

When a rat reticulum-cell sarcoma was transplanted after exposure to 3,000 r of x-rays, some of the transplants failed to grow (40). The hosts were immune to reinoculation of untreated grafts of the same neoplasm. Following regression of a lymphosarcoma grafted into riboflavin-deficient mice, subsequent tumor grafts did not grow (114). Regression of the same lymphosarcoma in pyridoxine-deficient mice did not induce immunity (113).

The first experiments on parabiosis in relation to susceptibility to transplanted leukemia revealed that if mice of a susceptible and of a resistant strain were united, inoculation of either parabiont with leukemic cells resulted in the development of leukemia by only the susceptible animal (30). Recent experiments utilizing the technic of parabiosis support the idea that humoral factors are involved in transplantation. Rats were made susceptible, by radiation with x-rays, to the heterologous transplantation of mouse leukemia. When an irradiated rat was united to a genetically similar untreated animal, the irradiated rat lost its susceptibility to the transplanted mouse leukemic cells (10). This experiment has been interpreted as demonstrating that humoral substances passing from the nonradiated to the radiated rat nullified the radiation-induced susceptibility.

Rats or mice which ordinarily do not develop leukemia when inoculated with leukemic cells from the same species may exhibit susceptibility if inoculated when relatively young with large doses of cells (45, 103). If genetic factors control the immunologic physiology determining transplantation, it might be concluded that transplants may be successful in otherwise resistant animals if they

are inoculated before the ability to form antibodies is well developed. Genetically determined resistance is relative, modified by the age of the recipient and the dose of leukemic cells.

A virus which is not to be considered a causative agent of mouse leukemia has been associated with several transfer lines and has been studied especially in relation to line I of the C58 strain (116). In mice inoculated with extracts of leukemic cells a lymphocytosis appeared, and the animals became sick 8–10 days after inoculation. All animals recovered and were immune to reinoculations of the virus. Leukemic cells can be freed from the virus by inoculation of cells into immune animals. The virus shortens the latent period of the transplanted disease, and influences the development of immunity against leukemic cells of line I.

Leukemic cells may contain a viral agent of perhaps another type (22). By culturing a lymphosarcoma, the mesenchymal were separated from the round cells. A lymphopenia resulted from the inoculation of either the leukemic round cells or extracts, but not from stromal mesenchymal cells. Animals were immune to subsequent inoculations.

In experiments demonstrating the transmission of leukemia by single intact cells it was found that leukemia did not result when the fragments of leukemic cells ruptured by microdissection were inoculated (33). Successful transmission of rat leukemia with cellular constituents alone has been described (112). Although the presence of intact cells was considered unlikely with the procedure used, many tumors appeared at the subcutaneous site of inoculation. Local appearance of tumors in the subcutaneous tissue makes cellular transmission a distinct probability.

Chemical agents may influence the development of transplanted leukemia. This will be discussed below under "Therapy."

Therapy

There are three fundamental types of rat or mouse leukemia which can be used in assaying the anti-leukemic effect of therapeutic agents—spontaneous, induced, transplanted. The spontaneous disease is more the counterpart of human leukemia than are the other two types. For various reasons, however, it has not been used in routine testing. First, an extremely large colony of animals is necessary to obtain a sufficient number of test animals; second, there is considerable variation in survival from case to case within even inbred strains; and third, the date of origin of the disease is uncertain, since the external manifestations of leukemia might not bear an exact time relation to the onset of malignant transformation. However, patients are treated only after the clinical onset of leukemia, and, if comparable material were to be used for experimental study, spontaneous mouse leukemia should be tested.

In the case of induced leukemia, agents might be assayed for effectiveness in two ways: first, to test their effect on inhibiting leukemogenesis; second, to test their ability to increase survival time once the disease has appeared. Induced leukemia has been used only infrequently to test agents (estrogens, androgens, x-rays) for either their anti-leukemogenic action (35, 58, 66)[3] or their effect on the established leukemic cell.

Transplanted leukemia is generally used in screening programs (39). It must be recognized that the transplanted leukemia is quite different from either the induced or spontaneous disease, in that the cells of the leukemic animal did not undergo their malignant transformation in the host, but are the progeny of cells which were malignant when introduced. Upon considering that it has been possible to immunize against transplanted, but not spontaneous, leukemia (80, 84), it should be realized that the leukemic cells of the two types of disease may be quite different. Transplantation may certainly affect the isoantigenic character of the leukemic cell.

After inoculation of leukemic cells a latent period exists before the animal may be considered leukemic—that is, a period before the inoculated leukemic cells have set up generalized foci of proliferation. Or, if a local tumor of lymphocytes is used for testing, as in the case of any other tumor, a latent period exists prior to the establishment of the tumor-host relation of the successful graft.

Although delaying or inhibiting the development of transplanted leukemia by instituting treatment within 1–4 days after transplantation may have significance, and although drugs demonstrating this effect have usually had the greatest effect upon human lesions, it must be recognized that inhibiting transplanted mouse leukemia is a far cry from successfully treating spontaneous mouse leukemia, not to mention human leukemia.

If an agent shows no inhibitory action against only certain transplanted mouse leukemias, this does not necessarily constitute evidence of its general ineffectiveness. Tremendous biological differences exist between the different transfer lines. Myeloid leukemia responds to certain drugs, lymphoid to others. It is encouraging that urethan and potassium arsenite, which are considered to influence human chronic myeloid leukemia, affect

[3] H. S. Kaplan and A. Kirschbaum, unpublished observations.

primarily the myeloid leukemias of mice, and similarly, the folic acid antagonists influence the acute lymphoid mouse leukemias (63). The essential findings of experimental therapy using transplanted leukemia are given below.

Radiomimetic drugs (nitrogen mustards, colchicine, urethan, alpha- and beta-peltatin, podophyllotoxin).—Administration of this group of chemicals results in profound cytological alterations in both normal hemopoietic tissue and malignant cells arising from this source. The question has been raised concerning the extent to which the action of these agents is direct, or mediated by way of the pituitary-adrenalcortical mechanism (5), since these agents are so much more effective on lymphoid than other tumors. A nonspecific noxious stimulus may induce regression of a transplanted lymphosarcoma (8). Retardation or regression of growth of lymphosarcoma 6C3HEDP was induced in either intact or adrenalectomized animals by the administration of one of the nitrogen mustards or urethan (6). Cortisone does, however, inhibit the growth of certain transplanted lymphoid tumors. Inhibition of growth may be obtained only with relatively large doses which are not compatible with extended survival (18). Fasting induced hypoplastic alteration in normal lymphoid organs only in the presence of the adrenals, whereas transplanted malignant lymphoid tissue was affected in adrenalectomized animals as well, indicating its independence of adrenal cortical control (1).

Increase in the survival time of mice with transplanted mouse leukemia was obtained with only certain of the nitrogen mustards, others being completely ineffective, indicating the chemical specificity of these agents (17).

Complete regression of a transplanted lymphosarcoma resulted from the administration of colchicine (9). In earlier experiments in which a colchicine-treated lymphosarcoma regressed, recurrence appeared and refractoriness to the drug developed (77). Not all mouse lymphosarcomas are sensitive to the action of this drug.[1]

Although urethan retards the development of certain transplanted myeloid, but not lymphoid, neoplastic growths of F strain mice (63), the effect in other strains of mice and rats is not confined to myeloid transfer lines (70, 90). Fully developed transplanted leukemia with widespread infiltration was not favorably influenced from the standpoint of survival.[1] The high white blood cell count dropped remarkably, the leukemic myeloid cells of the tissues showed a "shift to the right," and fewer mitotic figures were present (64). In affecting the normal hemopoietic tissues of the mouse and rat the lymphoid are more sensitive than the myeloid (47).

Temporary alkalosis follows urethan therapy. Administration of sodium bicarbonate and ammonium chloride to affect the acid-base balance temporarily did not alter the anti-leukemic effect (108). The effect of urethan on transplanted mouse leukemia is highly specific. Any change in the molecule either destroys or lessens the anti-leukemic action (106).

When carbonyl-labeled urethan was administered to both normal mice and animals with either leukemia or mammary cancer, the latter group retained more radioactivity in all tissues (13). There was a specific fixation of carbon for two groups in the urethan molecule to sperm, which has a high DNA content, suggesting that this compound combines with nuclear material (15).

Folic acid antagonists.—Following the report of effectiveness of folic acid antagonists in inducing remissions in human childhood leukemias, the inhibiting effects of these drugs were tested on transplanted mouse leukemias. If treatment is begun within 4 days after transplantation, the development of certain lines is delayed, whereas others are not affected (15, 63, 74).

Perhaps of more significance than the limited clinical benefits of therapy is the evidence that there develops a refractoriness of the leukemic cell per se to treatment. Sublines of transmitted leukemia were made resistant by passage for several generations through treated mice (19, 72). This resistance remained unchanged in thirty passages through treated animals (72). Morphologically, the sublines remained the same. Resistance to one 4-amino analog implies resistance to all, although the cells remain sensitive to the action of competitive antagonists of other chemical structure and to anti-leukemic agents such as alpha-peltatin.

Concentrations of folic acid antagonists which inhibit the growth of a susceptible subline are essential for the growth of variant sublines, indicating "dependence." Morphology, antigenicity, and transplantability of the transformed (resistant) and untreated sublines of resistant cells were similar (72).

The resistance of the leukemic cells to folic acid antagonists appears to be a stable and irreversible change. Either genetic mutation is induced with resultant resistant forms developing, or the susceptible cells of a varied population of leukemic cells are eliminated by treatment, the resistant forms being left behind to propagate the constituents of the resistant subline (72).

Refractoriness to potassium arsenite therapy

of a transplanted mouse myeloid leukemia could be induced by treatment of the host with the drug prior to transplantation of leukemic cells.[1] The host rather than the leukemic cell was responsible in this case for resistance to drug treatment. The possibility that the leukemic cells participate in this "fastness" has not been eliminated, although cells which have been passed through seven generations of treated hosts continue to respond to arsenic therapy.[1]

Hormones.—The growth of one line of mouse lymphosarcoma was retarded by the administration of cortisone (48). In another transfer line no increase in survival time was observed, but with 12.5 μg. given 8 times daily no leukemic infiltrations occurred (18). The white blood cell counts of leukemic mice dropped in conjunction with cortisone treatment; ACTH was less effective. The Murphy lymphosarcoma grew to a larger size in adrenalectomized than in intact rats; cortisone inhibited its growth if treatment was begun soon after transfer.[2] Compounds F, A, and corticosterone, as well as cortisone, each reduced the rate of growth of a transplanted mouse lymphoid tumor (120). Androgenic hormone inhibits the leukemogenic action of estrogenic hormone (38) and x-rays (35, 58).

Radiation.—Secondary effects of x-radiation were apparently involved in the inhibition of growth of a transplanted mouse lymphoid neoplasm (50). The effect obtained was maximum if the whole body as well as the tumor was radiated; radiation of the body exclusive of the tumor did not induce tumor regression. Administration of radioactive colloidal gold intraperitoneally results in the prolongation of life in certain transfer lines, but the distribution of gold is such (greatest amount in liver and spleen, relatively little in lymph nodes and bone marrow) that optimum radiation of leukemic infiltrations is not obtained.[1] The uptake of radioactive phosphorus by lymphomatous tissue is greater than by other tissues (102). Radioactive sodium, on the other hand, is not concentrated selectively in tumorous lymphoid tissue and is probably of no potential therapeutic value (28).

Retardation or inhibition of growth of transplanted leukemia and/or lymphosarcoma has been achieved by miscellaneous methods: injection of heterologous antibodies (94), ingestion of benzene (29), intravenous administration of aqueous suspensions of carcinogenic hydrocarbons (111), subjecting host to low body temperature (27), intravenous vaccinia (117), injection of testicular extract (3), pyridoxine and riboflavin deficiency (113, 114), injection of 8-azaguanine (60, 61, 71). The significance of these experiments is at present difficult to assess.

SUMMARY

1. Morphologic types of lymphoblastoma simulating those seen in man appear in rodents.

2. Interaction of genetic and extrinsic factors determine the time of appearance and type of lymphoblastoma. A nongenetic factor contributing towards resistance is transmitted from mother to offspring and may be supplied before birth or by nursing alone. Susceptibility to the development of "spontaneous" leukemia is not determined by genes identical with those involved in reactions to leukemogenic agents such as ionizing radiations, carcinogenic hydrocarbons, and estrogenic hormones. Specific genes may control susceptibility to each leukemogen. Leukemogenic agents may act synergistically in strains of mice susceptible to the independent action of each agent.

3. Leukemic cells of certain transfer lines may harbor a virus which is not an etiologic agent for leukemia, but which causes an illness hastening death from leukemia and alters the antigenicity of cells or the immune reaction of the host. Preliminary experiments have suggested that noncellular extracts of either leukemic tissue or of embryonic cells from a high leukemia strain may cause leukemia to appear "spontaneously" if injected into mice of a low-leukemia strain. Freezing and lyophilization of mouse leukemic cells render them inactive for transmitting leukemia.

4. Estrogen favors, androgen inhibits leukemogenesis in mice in certain situations. The leukemogenic effects of both x-rays and carcinogenic hydrocarbons may be potentiated by estrogenic hormone.

5. Susceptibility to the development of mouse leukemia may represent a property of the thymus, which serves not only as a primary locus for the development of the disease, but may influence its genesis in some unknown manner.

6. A humoral factor may be involved in the development of x-ray–induced leukemia. Radiation of the thymus alone, or of the whole body except the thymus, did not result in the induction of thymic tumors in animals which develop such neoplasms following whole body radiation. Since shielding of an extremity reduced the incidence of x-ray–induced thymoma, it appears that nonirradiated tissue may inactivate a humoral factor produced by radiation.

7. In a strain of mice susceptible to a particular leukemogenic agent, the younger the animal is at the time of exposure, the more the susceptibility is enhanced.

8. Nutritional factors such as caloric restriction or cystine deficiency may delay the development and reduce the incidence of spontaneous or carcinogen-induced leukemia. Pyridoxine or riboflavin deficiency are associated with the inhibition of growth of certain transplanted lymphosarcomas. It has been postulated that guanine is essential for the growth of neoplastic cells and that the growth of certain lymphosarcomas may be inhibited by the administration of 8-azaguanine, a guanine analog. The inhibitory effect of folic acid analogs on transplanted leukemia is attributed to interference with folic acid metabolism resulting in diminished nucleic acid synthesis.

9. Genetic factors control the transplantation of leukemic cells. The malignant transplantable elements are only the lymphoblastic round cells and not mesenchymal stromal elements. Antigen-antibody reactions may be involved in transplantability, since the gene presumably controls immunologic physiology. Homologous and heterologous immune sera may inactivate leukemic cells *in vitro*. Cytoplasmic constituents of leukemic cells are more highly antigenic than nuclei. Temporary growth of leukemic cells in foreign strains may be accelerated or inhibited by multiple prior injections of frozen, lyophilized tissue. Heterologous transplantation of leukemic cells can be accomplished by previous radiation of the foreign host, but not by inoculation into the anterior chamber of the eye. Humoral factors passing from normal to radiated rats joined by parabiosis may nullify radiation-induced susceptibility. Immunity towards transplanted leukemia can be passively transferred by inoculation of spleen or liver of immune animals. Resistance to homologous transplantation of leukemic cells may be overcome by inoculation of large doses of cells into very young animals.

10. Various agents may inhibit or delay the growth of certain lines of transplanted leukemic cells. Among these are "radiomimetic drugs," folic acid antagonists, cortical hormones, ionizing radiations, trivalent arsenic, benzene. Although the most active agents are those which have given the most encouraging clinical results, transplanted leukemia as generally used can at most be considered a helpful but not a critical testing medium. Wide variation exists in the response of different lines of leukemic cells to the same agent. Of greatest significance are the studies on refractoriness of leukemic cells and host to drug therapy.

REFERENCES

1. ADAMS, E., and WHITE, A. Influence of Fasting and Adrenalectomy on Normal and Malignant Lymphoid Tissue Composition. Proc. Soc. Exper. Biol. & Med., **75**: 590–95, 1950.
2. ALFIN-SLATER, R. B.; LARACK, A. M.; and PETERMANN, M. L. The Preparation and Properties of the Mitochondria and Submicroscopic Particles of Normal and Leukemic Mouse Organs. Cancer Research, **9**:215–16, 1949.
3. ARNESEN, K.; BUXTON, L.; and DULANEY, A. D. Effect of Testis Extract on Growth of a Transplanted Lymphosarcoma in AKm Mice. Proc. Soc. Exper. Biol. & Med., **71**:264–67, 1949.
4. ARNESEN, K.; GOLDSMITH, Y.; and DULANEY, A. D. Antigenic Properties of Nuclei Segregated from Spleens of Normal and Leukemic Mice. Cancer Research, **9**: 669–71, 1950.
5. BASS, A. D., and FEIGELSON, M. Response of Normal and Malignant Lymphoid Tissue to Non Specific Tissue Damage. Proc. Soc. Exper. Biol. & Med., **69**:339–41, 1948.
6. ———. The Response of Normal and Malignant Lymphoid Tissue to Methyl-Bis-(β-Chlorethyl)amine and Ethyl Carbamate (Urethane) in Adrenalectomized and Non-Adrenalectomized Mice. Cancer Research, **8**:503–9, 1948.
7. BASS, A. D., and FREEMAN, L. H. Response of Certain Mouse Tumors to Bis(β-Chlorethyl) sulfide (Mustard Gas). J. Nat. Cancer Inst., **7**:171–76, 1946.
8. ———. Regression of Lymphosarcoma Produced by Intraperitoneal Administration of 95% Ethyl Alcohol. Science, **107**:114–15, 1948.
9. BASS, A. D., and PROBERT, C. Response of a Transplantable Lymphosarcoma to Colchicine. Cancer Research, **10**:420–22, 1950.
10. BICHEL, J., and HOLM-JENSEN, I. Parabiosis and Resistance to Transplantation. I. The Influence of Parabiosis on the Growth of a Mouse Leukosis in Irradiated Rats. Acta pathol. microbiol. Scandinav., **24**:531–38, 1947.
11. BIESELE, J. J. Chromosomes in Lymphatic Leukemia of C58 Mice. Cancer Research, **7**:70–77, 1947.
12. BIESELE, J. J., and GASIC, G. Sex Hormone Effects on Chromosome Size in Leukemic and Normal Lymphocytes of C58 Mice. Cancer Research, **7**:65–69, 1947.
13. BRYAN, C. E.; SKIPPER, H. E.; and WHITE, L. Carbamates in the Chemotherapy of Leukemia. IV. The Distribution of Radioactivity in Tissues of Mice Following Injection of Carbonyl-labeled Urethane. J. Biol. Chem., **177**:941–50, 1949.
14. BURCHENAL, J. (in press).
15. BURCHENAL, J. H.; BURCHENAL, J. R.; KUSHIDA, M. N.; JOHNSTON, S. F.; and WILLIAMS, B. S. Studies on the Chemotherapy of Leukemia. II. The Effect of 4-Aminopteroylglutamic Acid and 4-Amino-N^{10}-Methyl-Pteroylglutamic Acid on Transplanted Mouse Leukemia. Cancer, **2**:113–18, 1949.
16. BURCHENAL, J. H.; JOHNSTON, S. F.; CREMER, M. A.; WEBBER, L. F.; and STOCK, C. C. Chemotherapy of Leukemia. V. Effects of 2,4,6-Triethylenimino-S-Triazine and Related Compounds on Transplanted Mouse Leukemia. Proc. Soc. Exper. Biol. & Med., **74**:708–12, 1950.
17. BURCHENAL, J. H., and RILEY, J. B. Nitrogen Mustards: The Relationship between Chemical Structure and Chemotherapeutic Activity. Cancer Research, **9**:553–54, 1949.
18. BURCHENAL, J. H.; STOCK, C. C.; and RHOADS, C. P. The Effects of Cortisone and ACTH on Transplanted Mouse Leukemia. Cancer Research, **10**:209, 1950.
19. BURCHENAL, J. H.; WEBBER, L. F.; MEIGS, G. M.; and BIEDLER, J. L. A Comparison of the Effects of 4-Amino-

N^{10}-Methyl-Pteroylglutamic Acid and 2,6-Diaminopurine upon Sensitive and Resistant Sublines of a Strain of Mouse Leukemia. Blood, **6**:337–43, 1951.

20. Claude, A. The Constitution of Mitochondria and Microsomes, and the Distribution of Nucleic Acid in the Cytoplasm of a Leukemic Cell. J. Exper. Med., **80**:19–29, 1944.
21. Cornman, I.; Skipper, H. E.; and Mitchell, J. H. The Fixation of Urethan Carbon Atoms in Sperm and in Resting and Rapidly Dividing Cells. Cancer Research, **11**:195–99, 1951.
22. de Bruyn, W. M. A Lymphopoenia-Causing Agent, Probably a Virus, Found in Mice after Injection with Tumor Tissue and with Cell-free Filtrates of Lymphosarcoma T86157(MB). Cancer Research, **9**:395–97, 1949.
23. De Bruyn, W. M.; Korteweg, R.; and van Waverin, K. Transplantable Mouse Lymphosarcoma T86157 (MB) Studied *in vivo*, *in vitro* and at Autopsy. Cancer Research, **9**:282–93, 1949.
24. Deringer, M., and Dunn, T. B. Mast Cell Neoplasia in Mice. J. Nat. Cancer Inst., **7**:289–98, 1947.
25. Dulaney, A. D., and Arnesen, K., Cytotoxic Action of Antisera to Cell Components of Normal and Leukemic Mouse Spleens. Proc. Soc. Exper. Biol. & Med., **72**:665–68, 1949.
26. Dulaney, A. D.; Goldsmith, Y.; Arnesen, K.; and Buxton, L. A Serological Study of Cytoplasmic Fractions from the Spleens of Normal and Leukemic Mice. Cancer Research, **9**:217–21, 1949.
27. Engelbreth-Holm, J., and Eltorm, H. Effect on Leukemia in Mice from Lowering of the Body Temperature. Acta path. et microbiol. Scandinav., **20**:346–59, 1943.
28. Evans, T. C., and Quimby, E.H. Studies on the Effects of Radioactive Sodium and of Roentgen Rays on Normal and Leukemic Mice. Am. J. Roentgenol., **55**:55–66, 1946.
29. Flory, C. M.; Steinhardt, I. D., and Furth, J. Further Observations on the Effect of Benzene on a Strain of Myeloid Chloroleukemia in Mice and on Changes Produced in the Leukemic Cells by the Chemical. Blood, **1**:367–78, 1946.
30. Furth, O. B.; Barnes, W. A.; and Brower, A. B. Studies on Resistance to Transmissible Leukemia in Mice by Means of Parabiosis. Arch. Path., **29**:163–74, 1940.
31. Furth, J., and Boon, M. C. Enhancement of Leukemogenic Action of Methylcholanthrene by Pre-irradiation with X-rays. Science, **98**:138–39, 1943.
32. Furth, J.; Boon, M. C.; and Kaliss, N. On the Genetic Character of Neoplastic Cells as Determined in Transplantation Experiments with Notes on the Somatic Mutation Theory. Cancer Research, **4**:1–10, 1944.
33. Furth, J., and Kahn, M.C. The Transmission of Leukemia of Mice with a Single Cell. Am. J. Cancer, **31**:276–82, 1937.
34. Gabrielson, R. M., and Syverton, J. T. The Effects of Freezing, Thawing, and Lyophilization upon the Transplantability of Mouse Leukemic Cells. Cancer Research, **11**:249, 1951.
35. Gardner, W. U. Ovarian and Lymphoid Tumors in Female Mice Subsequent to Roentgen-Ray Irradiation and Hormone Treatment. Proc. Soc. Exper. Biol. & Med., **75**:434–36, 1950.
36. ———. Steroid Hormones in the Induction of Cancer. Cancer Research, **7**:37–38, 1947.
37. Gardner, W. U., and Dougherty, T. F. The Leukemogenic Action of Estrogens in Hybrid Mice. Yale J. Biol. & Med., **17**:75–90, 1944.
38. Gardner, W. U.; Dougherty, T. F.; and Williams, W. L. Lymphoid Tumors in Mice Receiving Steroid Hormones. Cancer Research, **4**:73–87, 1944.
39. Geisse, N. C., and Kirschbaum, A. Transplanted Mouse Leukemia as a Test Object for the Evaluation of Chemotherapeutic Agents. Cancer Research, **10**:108–12, 1950.
40. Goldfeder, A. Induced Resistance in Inbred Homozygous Rats to a Lymphosarcoma Autogenous to the Strain. Proc. Soc. Exper. Biol. & Med., **59**:104–9, 1945.
41. Goldie, H., and Felix, M. D. Growth Characteristics of Free Tumor Cells Transferred Serially in the Peritoneal Fluid of the Mouse. Cancer Research, **11**:73–80, 1951.
42. Gorer, P. A. The Pathology of Malignant Histiocytoma (Reticuloendothelioma) of the Liver in Mice. Cancer Research, **6**:470–82, 1946.
43. ———. Role of Antibodies in Immunity to Transplanted Leukaemia in Mice. J. Path. & Bact., **54**:51–65, 1942.
44. Greenspan, E. M.; Leiter, J.; and Shear, M. J. Effect of Alpha-peltatin, Beta-peltatin and Podophyllotoxin on Lymphomas and Other Transplanted Tumors. J. Nat. Cancer Inst., **10**:1295–1333, 1950.
45. Gross, L. Susceptibility of Newborn Mice of an Otherwise Apparently "Resistant" Strain to Inoculation with Leukemia. Proc. Soc. Exper. Biol. & Med., **73**:246–48, 1950.
46. ———. "Spontaneous" Leukemia Developing in C3H Mice Following Inoculation in Infancy, and Ak-Leukemic Extracts, or Ak-Embryos. *Ibid.*, **76**:27–32, 1951.
47. Hawkins, J. A., and Murphy, J. B. The Effect of Ethyl Urethane Anesthesia on the Acid-Base Equilibrium and Cell Contents of the Blood. J. Exper. Med., **42**:609–18, 1925.
48. Heilman, F. R., and Kendall, E. C. The Influence of 11-Dehydro-17-Hydroxycorticosterone (Compound E) on the Growth of a Malignant Tumor in the Mouse. Endocrinology, **34**:416–20, 1944.
49. Hogreffe, G. Genetic Studies on Leukaemia in Mice. Brit. J. Cancer, **2**:108–14, 1948.
50. Hollcroft, J.; Lorenz, E.; and Hunstiger, H. Effects of Ionizing Radiations on a Transplanted Lymphosarcoma. Cancer Research, **10**:225, 1950.
51. Kaalund-Jorgensen, O. Experiments in Transmission of Leukoses from Mice to Roentgen-Irradiated Rats. Acta Radiol., **21**:483–99, 1940.
52. Kaplan, H. S. Observations on Radiation-Induced Lymphoid Tumors of Mice. Cancer Research, **7**:141–47, 1947.
53. ———. Comparative Susceptibility of the Lymphoid Tissues of Strain C57 Black Mice to the Induction of Lymphoid Tumors by Irradiation. J. Nat. Cancer Inst., **8**:191–97, 1948.
54. ———. Influence of Age on Susceptibility of Mice to the Development of Lymphoid Tumors after Irradiation. *Ibid.*, **9**:55–56, 1948.
55. ———. Preliminary Studies of the Effectiveness of Local Irradiation on the Induction of Lymphoid Tumors in Mice. *Ibid.*, **10**:267–70, 1949.
56. ———. Further Observations on Inhibition of Lymphoid Tumor Development by Shielding and Partial-Body Irradiation of Mice. Cancer Research, **11**:261–62, 1951.
57. Kaplan, H. S., and Brown, M. B. Effect on Lymphoid Tumor Incidence of Changes in Total Dose, Fractionation, and Periodicity of Whole-Body Roentgen Irradiation. Cancer Research, **11**:262, 1951.
58. ———. Inhibition by Testosterone of Radiation-induced Lymphoid Tumor Development in Intact and Castrate Male Mice. *Ibid.*, p. 262.
59. Kaplan, H. S.; Brown, M. B.; and Marder, S. N.

Adrenal Cortical Function and Lymphoid Tumor Incidence in Irradiated Mice. Cancer Research, **11**:262–63, 1951.

60. KIDDER, G. W.; DEWEY, V. C.; PARKS, R. E.; and WOODSIDE, G. L. Purine Metabolism in Tetrahymena and Its Relation to Malignant Cells in Mice. Science, **109**:511–14, 1949.
61. ———. Further Evidence on the Mode of Action of 8-Azaguanine (Guanazolo) in Tumor Inhibition. Cancer Research, **11**:204–11, 1951.
62. KIRSCHBAUM, A. Genetic and Certain Non-genetic Factors with Reference to Leukemia in the F Strain of Mice. Proc. Soc. Exper. Biol. & Med., **55**:147–49, 1944.
63. KIRSCHBAUM, A.; GEISSE, N. C.; JUDD, SISTER T.; and MEYER, L. M. Effect of Certain Folic Acid Antagonists on Transplanted Myeloid and Lymphoid Leukemias of the F Strain of Mice. Cancer Research, **10**:762–68, 1950.
64. KIRSCHBAUM, A., and LU, C. S. Effect of Urethane on Maturation of Leukocytes of Mouse Myelogenous Leukemia. Proc. Soc. Exper. Biol. & Med., **65**:62–63, 1947.
65. KIRSCHBAUM, A., and MIXER, H. W. Induction of Leukemia in Eight Inbred Stocks of Mice Varying in Susceptibility to the Spontaneous Disease. J. Lab. & Clin. Med., **32**:720–31, 1947.
66. KIRSCHBAUM, A.; SHAPIRO, J. R.; and MIXER, H. W. Synergistic Action of Estrogenic Hormone and X-rays in Inducing Thymic Lymphosarcoma of Mice. Proc. Soc. Exper. Biol. & Med., **72**:632–34, 1949.
67. KIRSCHBAUM, A., and STRONG, L. C. Leukemia in the F Strain of Mice: Observations on Cytology, General Morphology, and Transmission. Am. J. Cancer, **37**:400–13, 1939.
68. ———. Milk Influence and Leukemia in Mice. Proc. Soc. Exper. Biol. & Med., **51**:404–6, 1942.
69. LAW, L. W. Characterization of an Influence Affecting Growth of Transplantable Leukemias in Mice. Cancer Research, **4**:257–60, 1944.
70. LAW, L. W. Effect of Urethane on a Transplantable Acute Lymphoid Leukemia. Proc. Soc. Exper. Biol. & Med., **66**:158–61, 1947.
71. ———. Studies on the Effects of a Guanine Analog on Acute Lymphoid Leukemias of Mice. Cancer Research, **10**:186–90, 1950.
72. LAW, L. W., and BOYLE, P. J. Development of Resistance to Folic Acid Antagonists in a Transplantable Lymphoid Leukemia. Proc. Soc. Exper. Biol. & Med., **74**:599–602, 1950.
73. LAW, L. W.; BUNKER, L. E.; and NORRIS, B. A. Effect of Gonadectomy and Adrenalectomy on the Appearance and Incidence of Spontaneous Lymphoid Leukemia in C58 Mice. J. Nat. Cancer Inst., **8**:157–59, 1947.
74. LAW, L. W.; DUNN, T. B.; BOYLE, P. J.; and MILLER, J. H. Observations on the Effect of a Folic Acid Antagonist on Transplantable Lymphoid Leukemias in Mice. J. Nat. Cancer Inst., **10**:179–92, 1949.
75. LAW, L. W., and MILLER, J. H. The Influence of Thymectomy on the Incidence of Carcinogen-induced Leukemia in Strain DBA Mice. J. Nat. Cancer Inst., **11**:425–37, 1950.
76. ———. Observations on the Effect of Thymectomy on Spontaneous Leukemias in Mice of the High-Leukemic Strains, RIL and C58. *Ibid.*, pp. 253–62.
77. LITS, F. J.; KIRSCHBAUM, A.; and STRONG, L. C. Action of Colchicine on a Malignant Lymphoid Neoplasm in Mice of an Inbred Strain. Proc. Soc. Exper. Biol. & Med., **38**:555–57, 1938.
78. LOWENHAUPT, E. Splenic Lymphosarcomas in Rats Bearing Intrasplenic Implants of Butter Yellow. Cancer Research, **9**:121–26, 1949.
79. LUSHBAUGH, C. C., and STEINER, P. E. Intraocular Transplantation of Malignant Lymphomas of the Mouse, Dog, and Man in Heterologous Species. Cancer Research, **9**:299–305, 1949.
80. MACDOWELL, E. C.; POTTER, J. S.; and TAYLOR, M. J. The Influence of Transplantation upon Immunological Properties of Leukemic Cells. Proc. Nat. Acad. Sc., **25**:416–20, 1939.
81. ———. Mouse Leukemia. XII. The Role of Genes in Spontaneous Cases. Cancer Research, **5**:65–83, 1945.
82. MACDOWELL, E. C., and RICHTER, M. N. Mouse Leukemia. IX. The Role of Heredity in Spontaneous Cases. Arch. Path., **20**:709–24, 1935.
83. MACDOWELL, E. C., and TAYLOR, M. J. Mouse Leukemia. XIII. A Maternal Influence that Lowers the Incidence of Spontaneous Cases. Proc. Soc. Exper. Biol. & Med., **68**:571–77, 1948.
84. MACDOWELL, E. C.; TAYLOR, M. J.; and POTTER, J. S. Immunization of Mice Naturally Susceptible to a Transplantable Leukemia. Proc. Soc. Exper. Biol. & Med., **32**:84–86, 1934.
85. MCENDY, D. P.; BOON, M. C.; and FURTH, J. On the Role of Thymus, Spleen, and Gonads in the Development of Leukemia in a High-Leukemia Stock of Mice. Cancer Research, **4**:377–83, 1944.
86. MILLER, R. A., and TAYLOR, M. J. A Concomitant Change in Mitochondria and Virulence of a Transplanted Lymphoid Leukemia. Proc. Soc. Exper. Biol. & Med., **68**:336–39, 1948.
87. MOON, H. D.; SIMPSON, M. E.; LI, C. H.; and EVANS, H. M. Neoplasms in Rats Treated with Pituitary Growth Hormone. I. Pulmonary and Lymphatic Tissues. Cancer Research, **10**:297–308, 1950.
88. MURPHY, J. B. The Effect of Castration, Theelin, and Testosterone on the Incidence of Leukemia in a Rockefeller Institute Strain of Mice. Cancer Research, **4**:622–24, 1944.
89. MURPHY, J. B., and STURM, E. Effect of Adrenal Cortical and Pituitary Adrenotropic Hormones on Transplanted Leukemia in Rats. Science, **99**:303, 1944.
90. ———. The Inhibiting Effect of Ethyl Urethane on the Development of Lymphatic Leukemia in Rats. Cancer Research, **7**:417–20, 1947.
91. ———. The Effect of Diethylstilbestrol on the Incidence of Leukemia in Male Mice of the Rockefeller Institute Leukemia Strain (R.I.L.). *Ibid.*, **9**:88–89, 1949.
92. NETTLESHIP, A. Influence of Age on the Growth of Lymphomas. Am. J. Path., **21**:147–59, 1945.
93. ———. Growth of a Mouse Lymphoma Compared to Normal Tissue Growth. *Ibid.*, pp. 167–69.
94. ———. Regression Produced in the Murphy Lymphosarcoma by the Injection of Heterologous Antibodies. *Ibid.*, pp. 527–41.
95. PETERMANN, M. L., and MASON, E. J. Nucleic Acid Content of Chromosomes of Normal and Leukemic Mouse Spleen. Proc. Soc. Exper. Biol. & Med., **69**:542–44, 1948.
96. POTTER, J. S.; TAYLOR, M. J.; and MACDOWELL, E. C. Transfer of Acquired Resistance to Transplantable Leukemia in Mice. Proc. Soc. Exper. Biol. & Med., **37**:655–56, 1938.
97. RASK-NIELSEN, R. Investigations into the Varying Manifestations of Leukaemic Lesions Following Injections of 9:10-Dimethyl-1:2-Benzanthracene into Different Subcutaneous Sites in Street Mice. Brit. J. Cancer, **3**:549–56, 1949.
98. ———. On the Susceptibility of the Thymus, Lung,

N^{10}-Methyl-Pteroylglutamic Acid and 2,6-Diaminopurine upon Sensitive and Resistant Sublines of a Strain of Mouse Leukemia. Blood, **6**:337–43, 1951.

20. Claude, A. The Constitution of Mitochondria and Microsomes, and the Distribution of Nucleic Acid in the Cytoplasm of a Leukemic Cell. J. Exper. Med., **80**:19–29, 1944.
21. Cornman, I.; Skipper, H. E.; and Mitchell, J. H. The Fixation of Urethan Carbon Atoms in Sperm and in Resting and Rapidly Dividing Cells. Cancer Research, **11**:195–99, 1951.
22. de Bruyn, W. M. A Lymphopoenia-Causing Agent, Probably a Virus, Found in Mice after Injection with Tumor Tissue and with Cell-free Filtrates of Lymphosarcoma T86157(MB). Cancer Research, **9**:395–97, 1949.
23. De Bruyn, W. M.; Korteweg, R.; and van Waverin, K. Transplantable Mouse Lymphosarcoma T86157 (MB) Studied *in vivo*, *in vitro* and at Autopsy. Cancer Research, **9**:282–93, 1949.
24. Deringer, M., and Dunn, T. B. Mast Cell Neoplasia in Mice. J. Nat. Cancer Inst., **7**:289–98, 1947.
25. Dulaney, A. D., and Arnesen, K., Cytotoxic Action of Antisera to Cell Components of Normal and Leukemic Mouse Spleens. Proc. Soc. Exper. Biol. & Med., **72**:665–68, 1949.
26. Dulaney, A. D.; Goldsmith, Y.; Arnesen, K.; and Buxton, L. A Serological Study of Cytoplasmic Fractions from the Spleens of Normal and Leukemic Mice. Cancer Research, **9**:217–21, 1949.
27. Engelbreth-Holm, J., and Eltorm, H. Effect on Leukemia in Mice from Lowering of the Body Temperature. Acta path. et microbiol. Scandinav., **20**:346–59, 1943.
28. Evans, T. C., and Quimby, E.H. Studies on the Effects of Radioactive Sodium and of Roentgen Rays on Normal and Leukemic Mice. Am. J. Roentgenol., **55**:55–66, 1946.
29. Flory, C. M.; Steinhardt, I. D., and Furth, J. Further Observations on the Effect of Benzene on a Strain of Myeloid Chloroleukemia in Mice and on Changes Produced in the Leukemic Cells by the Chemical. Blood, **1**:367–78, 1946.
30. Furth, O. B.; Barnes, W. A.; and Brower, A. B. Studies on Resistance to Transmissible Leukemia in Mice by Means of Parabiosis. Arch. Path., **29**:163–74, 1940.
31. Furth, J., and Boon, M. C. Enhancement of Leukemogenic Action of Methylcholanthrene by Pre-irradiation with X-rays. Science, **98**:138–39, 1943.
32. Furth, J.; Boon, M. C.; and Kaliss, N. On the Genetic Character of Neoplastic Cells as Determined in Transplantation Experiments with Notes on the Somatic Mutation Theory. Cancer Research, **4**:1–10, 1944.
33. Furth, J., and Kahn, M.C. The Transmission of Leukemia of Mice with a Single Cell. Am. J. Cancer, **31**:276–82, 1937.
34. Gabrielson, R. M., and Syverton, J. T. The Effects of Freezing, Thawing, and Lyophilization upon the Transplantability of Mouse Leukemic Cells. Cancer Research, **11**:249, 1951.
35. Gardner, W. U. Ovarian and Lymphoid Tumors in Female Mice Subsequent to Roentgen-Ray Irradiation and Hormone Treatment. Proc. Soc. Exper. Biol. & Med., **75**:434–36, 1950.
36. ———. Steroid Hormones in the Induction of Cancer. Cancer Research, **7**:37–38, 1947.
37. Gardner, W. U., and Dougherty, T. F. The Leukemogenic Action of Estrogens in Hybrid Mice. Yale J. Biol. & Med., **17**:75–90, 1944.
38. Gardner, W. U.; Dougherty, T. F.; and Williams, W. L. Lymphoid Tumors in Mice Receiving Steroid Hormones. Cancer Research, **4**:73–87, 1944.
39. Geisse, N. C., and Kirschbaum, A. Transplanted Mouse Leukemia as a Test Object for the Evaluation of Chemotherapeutic Agents. Cancer Research, **10**:108–12, 1950.
40. Goldfeder, A. Induced Resistance in Inbred Homozygous Rats to a Lymphosarcoma Autogenous to the Strain. Proc. Soc. Exper. Biol. & Med., **59**:104–9, 1945.
41. Goldie, H., and Felix, M. D. Growth Characteristics of Free Tumor Cells Transferred Serially in the Peritoneal Fluid of the Mouse. Cancer Research, **11**:73–80, 1951.
42. Gorer, P. A. The Pathology of Malignant Histiocytoma (Reticuloendothelioma) of the Liver in Mice. Cancer Research, **6**:470–82, 1946.
43. ———. Role of Antibodies in Immunity to Transplanted Leukaemia in Mice. J. Path. & Bact., **54**:51–65, 1942.
44. Greenspan, E. M.; Leiter, J.; and Shear, M. J. Effect of Alpha-peltatin, Beta-peltatin and Podophyllotoxin on Lymphomas and Other Transplanted Tumors. J. Nat. Cancer Inst., **10**:1295–1333, 1950.
45. Gross, L. Susceptibility of Newborn Mice of an Otherwise Apparently "Resistant" Strain to Inoculation with Leukemia. Proc. Soc. Exper. Biol. & Med., **73**:246–48, 1950.
46. ———. "Spontaneous" Leukemia Developing in C3H Mice Following Inoculation in Infancy, and Ak-Leukemic Extracts, or Ak-Embryos. *Ibid.*, **76**:27–32, 1951.
47. Hawkins, J. A., and Murphy, J. B. The Effect of Ethyl Urethane Anesthesia on the Acid-Base Equilibrium and Cell Contents of the Blood. J. Exper. Med., **42**:609–18, 1925.
48. Heilman, F. R., and Kendall, E. C. The Influence of 11-Dehydro-17-Hydroxycorticosterone (Compound E) on the Growth of a Malignant Tumor in the Mouse. Endocrinology, **34**:416–20, 1944.
49. Hogreffe, G. Genetic Studies on Leukaemia in Mice. Brit. J. Cancer, **2**:108–14, 1948.
50. Hollcroft, J.; Lorenz, E.; and Hunstiger, H. Effects of Ionizing Radiations on a Transplanted Lymphosarcoma. Cancer Research, **10**:225, 1950.
51. Kaalund-Jorgensen, O. Experiments in Transmission of Leukoses from Mice to Roentgen-Irradiated Rats. Acta Radiol., **21**:483–99, 1940.
52. Kaplan, H. S. Observations on Radiation-Induced Lymphoid Tumors of Mice. Cancer Research, **7**:141–47, 1947.
53. ———. Comparative Susceptibility of the Lymphoid Tissues of Strain C57 Black Mice to the Induction of Lymphoid Tumors by Irradiation. J. Nat. Cancer Inst., **8**:191–97, 1948.
54. ———. Influence of Age on Susceptibility of Mice to the Development of Lymphoid Tumors after Irradiation. *Ibid.*, **9**:55–56, 1948.
55. ———. Preliminary Studies of the Effectiveness of Local Irradiation on the Induction of Lymphoid Tumors in Mice. *Ibid.*, **10**:267–70, 1949.
56. ———. Further Observations on Inhibition of Lymphoid Tumor Development by Shielding and Partial-Body Irradiation of Mice. Cancer Research, **11**:261–62, 1951.
57. Kaplan, H. S., and Brown, M. B. Effect on Lymphoid Tumor Incidence of Changes in Total Dose, Fractionation, and Periodicity of Whole-Body Roentgen Irradiation. Cancer Research, **11**:262, 1951.
58. ———. Inhibition by Testosterone of Radiation-induced Lymphoid Tumor Development in Intact and Castrate Male Mice. *Ibid.*, p. 262.
59. Kaplan, H. S.; Brown, M. B.; and Marder, S. N.

Adrenal Cortical Function and Lymphoid Tumor Incidence in Irradiated Mice. Cancer Research, **11**:262–63, 1951.

60. KIDDER, G. W.; DEWEY, V. C.; PARKS, R. E.; and WOODSIDE, G. L. Purine Metabolism in Tetrahymena and Its Relation to Malignant Cells in Mice. Science, **109**:511–14, 1949.
61. ———. Further Evidence on the Mode of Action of 8-Azaguanine (Guanazolo) in Tumor Inhibition. Cancer Research, **11**:204–11, 1951.
62. KIRSCHBAUM, A. Genetic and Certain Non-genetic Factors with Reference to Leukemia in the F Strain of Mice. Proc. Soc. Exper. Biol. & Med., **55**:147–49, 1944.
63. KIRSCHBAUM, A.; GEISSE, N. C.; JUDD, SISTER T.; and MEYER, L. M. Effect of Certain Folic Acid Antagonists on Transplanted Myeloid and Lymphoid Leukemias of the F Strain of Mice. Cancer Research, **10**:762–68, 1950.
64. KIRSCHBAUM, A., and LU, C. S. Effect of Urethane on Maturation of Leukocytes of Mouse Myelogenous Leukemia. Proc. Soc. Exper. Biol. & Med., **65**:62–63, 1947.
65. KIRSCHBAUM, A., and MIXER, H. W. Induction of Leukemia in Eight Inbred Stocks of Mice Varying in Susceptibility to the Spontaneous Disease. J. Lab. & Clin. Med., **32**:720–31, 1947.
66. KIRSCHBAUM, A.; SHAPIRO, J. R.; and MIXER, H. W. Synergistic Action of Estrogenic Hormone and X-rays in Inducing Thymic Lymphosarcoma of Mice. Proc. Soc. Exper. Biol. & Med., **72**:632–34, 1949.
67. KIRSCHBAUM, A., and STRONG, L. C. Leukemia in the F Strain of Mice: Observations on Cytology, General Morphology, and Transmission. Am. J. Cancer, **37**:400–13, 1939.
68. ———. Milk Influence and Leukemia in Mice. Proc. Soc. Exper. Biol. & Med., **51**:404–6, 1942.
69. LAW, L. W. Characterization of an Influence Affecting Growth of Transplantable Leukemias in Mice. Cancer Research, **4**:257–60, 1944.
70. LAW, L. W. Effect of Urethane on a Transplantable Acute Lymphoid Leukemia. Proc. Soc. Exper. Biol. & Med., **66**:158–61, 1947.
71. ———. Studies on the Effects of a Guanine Analog on Acute Lymphoid Leukemias of Mice. Cancer Research, **10**:186–90, 1950.
72. LAW, L. W., and BOYLE, P. J. Development of Resistance to Folic Acid Antagonists in a Transplantable Lymphoid Leukemia. Proc. Soc. Exper. Biol. & Med., **74**:599–602, 1950.
73. LAW, L. W.; BUNKER, L. E.; and NORRIS, B. A. Effect of Gonadectomy and Adrenalectomy on the Appearance and Incidence of Spontaneous Lymphoid Leukemia in C58 Mice. J. Nat. Cancer Inst., **8**:157–59, 1947.
74. LAW, L. W.; DUNN, T. B.; BOYLE, P. J.; and MILLER, J. H. Observations on the Effect of a Folic Acid Antagonist on Transplantable Lymphoid Leukemias in Mice. J. Nat. Cancer Inst., **10**:179–92, 1949.
75. LAW, L. W., and MILLER, J. H. The Influence of Thymectomy on the Incidence of Carcinogen-induced Leukemia in Strain DBA Mice. J. Nat. Cancer Inst., **11**:425–37, 1950.
76. ———. Observations on the Effect of Thymectomy on Spontaneous Leukemias in Mice of the High-Leukemic Strains, RIL and C58. *Ibid.*, pp. 253–62.
77. LITS, F. J.; KIRSCHBAUM, A.; and STRONG, L. C. Action of Colchicine on a Malignant Lymphoid Neoplasm in Mice of an Inbred Strain. Proc. Soc. Exper. Biol. & Med., **38**:555–57, 1938.
78. LOWENHAUPT, E. Splenic Lymphosarcomas in Rats Bearing Intrasplenic Implants of Butter Yellow. Cancer Research, **9**:121–26, 1949.
79. LUSHBAUGH, C. C., and STEINER, P. E. Intraocular Transplantation of Malignant Lymphomas of the Mouse, Dog, and Man in Heterologous Species. Cancer Research, **9**:299–305, 1949.
80. MACDOWELL, E. C.; POTTER, J. S.; and TAYLOR, M. J. The Influence of Transplantation upon Immunological Properties of Leukemic Cells. Proc. Nat. Acad. Sc., **25**:416–20, 1939.
81. ———. Mouse Leukemia. XII. The Role of Genes in Spontaneous Cases. Cancer Research, **5**:65–83, 1945.
82. MACDOWELL, E. C., and RICHTER, M. N. Mouse Leukemia. IX. The Role of Heredity in Spontaneous Cases. Arch. Path., **20**:709–24, 1935.
83. MACDOWELL, E. C., and TAYLOR, M. J. Mouse Leukemia. XIII. A Maternal Influence that Lowers the Incidence of Spontaneous Cases. Proc. Soc. Exper. Biol. & Med., **68**:571–77, 1948.
84. MACDOWELL, E. C.; TAYLOR, M. J.; and POTTER, J. S. Immunization of Mice Naturally Susceptible to a Transplantable Leukemia. Proc. Soc. Exper. Biol. & Med., **32**:84–86, 1934.
85. MCENDY, D. P.; BOON, M. C.; and FURTH, J. On the Role of Thymus, Spleen, and Gonads in the Development of Leukemia in a High-Leukemia Stock of Mice. Cancer Research, **4**:377–83, 1944.
86. MILLER, R. A., and TAYLOR, M. J. A Concomitant Change in Mitochondria and Virulence of a Transplanted Lymphoid Leukemia. Proc. Soc. Exper. Biol. & Med., **68**:336–39, 1948.
87. MOON, H. D.; SIMPSON, M. E.; LI, C. H.; and EVANS, H. M. Neoplasms in Rats Treated with Pituitary Growth Hormone. I. Pulmonary and Lymphatic Tissues. Cancer Research, **10**:297–308, 1950.
88. MURPHY, J. B. The Effect of Castration, Theelin, and Testosterone on the Incidence of Leukemia in a Rockefeller Institute Strain of Mice. Cancer Research, **4**:622–24, 1944.
89. MURPHY, J. B., and STURM, E. Effect of Adrenal Cortical and Pituitary Adrenotropic Hormones on Transplanted Leukemia in Rats. Science, **99**:303, 1944.
90. ———. The Inhibiting Effect of Ethyl Urethane on the Development of Lymphatic Leukemia in Rats. Cancer Research, **7**:417–20, 1947.
91. ———. The Effect of Diethylstilbestrol on the Incidence of Leukemia in Male Mice of the Rockefeller Institute Leukemia Strain (R.I.L.). *Ibid.*, **9**:88–89, 1949.
92. NETTLESHIP, A. Influence of Age on the Growth of Lymphomas. Am. J. Path., **21**:147–59, 1945.
93. ———. Growth of a Mouse Lymphoma Compared to Normal Tissue Growth. *Ibid.*, pp. 167–69.
94. ———. Regression Produced in the Murphy Lymphosarcoma by the Injection of Heterologous Antibodies. *Ibid.*, pp. 527–41.
95. PETERMANN, M. L., and MASON, E. J. Nucleic Acid Content of Chromosomes of Normal and Leukemic Mouse Spleen. Proc. Soc. Exper. Biol. & Med., **69**:542–44, 1948.
96. POTTER, J. S.; TAYLOR, M. J.; and MACDOWELL, E. C. Transfer of Acquired Resistance to Transplantable Leukemia in Mice. Proc. Soc. Exper. Biol. & Med., **37**:655–56, 1938.
97. RASK-NIELSEN, R. Investigations into the Varying Manifestations of Leukaemic Lesions Following Injections of 9:10-Dimethyl-1:2-Benzanthracene into Different Subcutaneous Sites in Street Mice. Brit. J. Cancer, **3**:549–56, 1949.
98. ———. On the Susceptibility of the Thymus, Lung,

Subcutaneous and Mammary Tissues in Strain Street Mice to Direct Application of Small Doses of Four Different Carcinogenic Hydrocarbons. *Ibid.*, **4**:108–16, 1950.

99. ———. Local and Remote Tumours in Strain Street Mice following Subcutaneous Injection of Large Doses of Four Different Carcinogenic Hydrocarbons. *Ibid.*, pp. 124–32.
100. Rask-Nielsen, R., and Gormsen, H. Spontaneous and Induced Plasma-Cell Neoplasia in a Strain of Mice. Cancer, **4**:387–97, 1951.
101. Saxton, J. A.; Boon, M. C.; and Furth, J. Observations on the Inhibition of Development of Spontaneous Leukemia in Mice by Underfeeding. Cancer Research, **4**: 401–9, 1944.
102. Scott, K. G. Metabolic Studies on Leukemic Mice with the Aid of Radioactive Phosphorus. Cancer Research, **5**:365–67, 1945.
103. Shay, H.; Gruenstein, M.; and Glazer, L. Uniform Transfer to Random Bred Rats of Lymphatic Leukemia Induced by Gastric Instillation of Methylcholanthrene. Proc. Soc. Exper. Biol. & Med., **75**:753–56, 1950.
104. Shay, H.; Gruenstein, M.; Marx, H. E.; and Glazer, L. The Development of Lymphatic and Myelogenous Leukemia in Wistar Rats Following Gastric Instillation of Methylcholanthrene. Cancer Research, **11**:29–34, 1951.
105. Shay, H.; Weinhouse, S.; Gruenstein, M.; Marx, H. E.; Friedman, B. Development of Malignant Lymphoma in Some of the Young Rats Suckled by Mothers Receiving Methylcholanthrene by Stomach Tube Only during the Lactation Period. Cancer, **3**:891–95, 1950.
106. Skipper, H. E., and Bryan, C. E. Carbamates in the Chemotherapy of Leukemia. III. The Relationship between Chemical Structure and Anti-Leukemic Action of a Series of Urethan Derivatives. J. Nat. Cancer Inst., **9**: 391–97, 1949.
107. Skipper, H. E., and Burchenal, J. H. The Nucleic Acid Inhibiting Action of 4-Amino-N^{10}-Methylpteroylglutamic Acid in Mice with a Sensitive and Resistant Strain of Leukemia. Cancer Research, **11**:229–31, 1951.
108. Skipper, H. E., and Chapman, J. B. Carbamates in the Chemotherapy of Leukemia. VI. The Influence of Urethan and Blood Acid-Base Equilibrium on Mouse Chloroleukemia 1394. Cancer Research, **9**:158–61, 1949.
109. Snell, G. D.; Cloudman, A. M.; Failor, E.; and Douglass, P. Inhibition and Stimulation of Tumor Homoiotransplants by Prior Injections of Lyophilized Tumor Tissue. J. Nat. Cancer Inst., **6**:303–16, 1946.
110. Snell, G. D.; Cloudman, A. M.; and Woodworth, E. Tumor Immunity in Mice, Induced with Lyophilized Tissue as Influenced by Tumor Strain, Host Strain, Source of Tissue, and Dosage. Cancer Research, **8**:429–37, 1948.
111. Stamer, S., and Engelbreth-Holm, J. Influence of Carcinogenic Hydrocarbon upon Transplanted Leukemia. Acta path. et microbiol. Scandinav., **20**:360–71, 1948.
112. Stasney, J.; Cantarow, A.; and Paschkis, K. E. Production of Neoplasms by Injection of Fractions of Mammalian Neoplasms. Cancer Research, **10**:775–82, 1950.
113. Stoerk, H. C. The Regression of Lymphosarcoma Implants in Pyridoxine-Deficient Mice. J. Biol. Chem., **171**:437–38, 1947.
114. Stoerk, H. C., and Emerson, G. A. Complete Regression of Lymphosarcoma Implants Following Temporary Induction of Riboflavin Deficiency in Mice. Proc. Soc. Exper. Biol. & Med., **70**:703–4, 1949.
115. Sturm, E., and Murphy, J. B. The Effect of Adrenalectomy on the Susceptibility of Rats to a Transplantable Leukemia. Cancer Research, **4**:384–88, 1944.
116. Taylor, M. J., and MacDowell, E. C. Mouse Leukemia. XIV. Freeing Transplanted Line I from a Contaminating Virus. Cancer Research, **9**:144–49, 1949.
117. Turner, J. C., and Mulliken, B. Effects of Intravenous Vaccinia in Mice with Sarcoma 180 or Leukemia 9417. Cancer, **3**:354–60, 1950.
118. Werder, A. A.; Kirschbaum, A.; and Syverton, J. T. The Effects *in vitro* of Specific Antibodies on the Cells of a Transplantable Mouse Leukemia. Cancer Research, **10**:248, 1950.
119. White, J.; White, F. R.; and Mider, G. B. Effect of Diets Deficient in Certain Amino Acids on the Induction of Leukemia in DBA Mice. J. Nat. Cancer Inst., **7**:199–202, 1947.
120. Woolley, G. W. Cortisone, Related Steroids, and Transplanted Tumors of the Mouse. Cancer Research, **11**:291, 1951.

Gastric Absorption of 3,4-Benzpyrene

I. The Effect of Physiological Processes on Absorption

PENTTI ERMALA, KAI SETÄLÄ, AND PER EKWALL

(Department of Anatomy and Department of Roentgenology, Second Medical Clinic, University of Helsinki, and the Institute of Physical Chemistry, Åbo Akademi, Finland)

Adenocarcinoma of the glandular region of the stomach has not been induced by feeding the fat-soluble carcinogenic hydrocarbons to mice and rats, although carcinoma of the forestomach and/or of the small intestine may occur (1, 4, 23, 28). It has been suggested that the glandular mucosa of the stomach is relatively resistant to tumor induction and that there may exist a "protective mucous barrier" which prevents contact between the carcinogen and the mucosal cells (2, 5, 11, 13, 15, 16, 28, 30). However, the glandular mucosa is susceptible to the action of carcinogenic agents when introduced directly into the wall of the stomach (12, 22, 29). In addition, gastric adenocarcinomas are reported to be induced by feeding heated lipids (6, 20, 21, 31), although similar experiments by others have failed (2, 15).

In a study of experimental gastric carcinogenesis, the gastric absorption of carcinogenic hydrocarbons requires investigation. The role of the stomach in digestion and the differences between gastric and intestinal absorption, as well as the various factors influencing these processes, have been largely neglected in the study of experimental gastric carcinogenesis.

It has been shown previously (24, 25) that 3,4-benzpyrene in certain vehicles penetrates the various coats of the stomach. Also, the fluorescent constituents of tobacco tar are absorbed in the gastrointestinal tract (27).

In the present study the absorption of 3,4-benzpyrene in normal mice and cats has been investigated in different parts of the gastrointestinal tract, taking into account the physiologic background of absorption of ingested material.

MATERIALS AND METHODS

The rate of absorption of 3,4-benzpyrene (BP) (Eastman Kodak Co., Rochester, N.Y.) in various solvents, after gastric instillation, was studied by determining the blue fluorescence of the material with the fluorescence microscope technic (Reichert Lux UV with a Philora HPW 125-watt mercury vapor lamp). The intensity and the penetration power of the blue fluorescence was estimated by using an arbitrary gradation (cf. Fig. 2). However, spectroscopic estimations were not made, and therefore it is unknown whether the blue fluorescence in the tissues depends on unchanged BP or on its blue fluorescent metabolites. Bearing this in mind, reference is made only to the absorption of BP in the present work.

About 600 healthy adult stock mice of both sexes, employed for several years in our studies, were used. Some experiments were conducted with C3H mice with similar results. Additional investigations were carried out with young cats, weighing 250–350 gm. All the animals were kept under similar laboratory conditions, and the diet was sufficient and balanced, particularly with respect to vitamins A and B. No pathologic changes, including spontaneous tumors, were encountered. The animals fasted at least 12 hours before administration of BP, as well as during the entire experiment. Thus, the stomach was nearly empty, and dilution of the solutions instilled was prevented, except that due to the normal gastric secretion. The amount of solutions administered by stomach tube was 0.2 cc. per mouse and 2.0 cc. per cat. The animals were killed by decapitation 5, 15, 30, or 60 minutes, or 24–48 hours after feeding. The organs, after fixation in 10 per cent neutral formalin solution (from 1 to 10 hours), were cut on the freezing microtome at 10 μ thickness, mounted in anhydrous glycerol, and examined (at least 5 hours after autopsy). Control observations were made on unfixed and unmounted specimens. Immersion of the organs in the fixative solution for less than about 10 hours produced no essential changes in the localization and in the intensity of the blue fluorescence in the tissues. Other specimens were imbedded in paraffin and stained in the usual manner for comparison.

Because carcinogenic hydrocarbons are fat-soluble, it seems likely that the mechanism of their

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absorption is linked to that of lipids in general. The following vehicles were used: (*a*) natural fats: olive oil and arachidis oil; (*b*) natural fats, emulsified in natural ox bile, with controls of (*c*) natural ox bile only; (*d*) lecithin, representing natural phosphatides. Parallel experiments were made using (*e*) unsaponifiable light petrolatum and (*f*) emulsions of light petrolatum stabilized with Aerosol OT, or with sodium taurocholate (17). In addition, a great number of substances (*g*), possessing simultaneously lipo- and hydrophilic properties, were examined, both in aqueous and in nonaqueous solution (8, 24, 25). The physico-chemical properties are discussed in the following report (7).

The BP concentration varied from 0.5 to 0.01 per cent, owing to the differences in the solubility of BP in various solvents. For adequate comparison, results have been corrected to standard conditions.

To determine the possible importance of individual variation in the amount of gastric secretion to the rate of absorption, histamine experiments were carried out: 0.2 cc. of histamine solution, containing 0.5 mg. of histamine hydrochloride *pro dosi*, was injected subcutaneously in mice every 15 minutes during this experiment.

RESULTS

Stomach of the Mouse

Only the most important observations made on the blue fluorescence in the wall of the mouse stomach 30 minutes after gastric instillation of BP in various mediators are presented in the following (cf. Figs. 1, 2, for orientation).

Glandular stomach.—BP, dissolved in natural fats or in light petrolatum, did not cause any visible blue fluorescence in the glandular mucosa, either in the highest concentration (0.5 per cent) used, or when the animals were killed after 6 hours, during which time only a comparatively small amount of the fat solution had entered the intestine, and thus the surface of the glandular mucosa had been in close contact with the BP solution all that time. These results agree with the observations made in feeding experiments which have failed to produce adenocarcinomas, and in which natural fats have been mainly employed as solvent for the hydrocarbon.

With all the other vehicles, signs of absorption of BP were observed in varying degrees. The intensity of fluorescence as well as the penetration power varied considerably in different groups, but the blue fluorescence was qualitatively similar in all cases.

BP, dissolved in natural fats (e.g., in arachidis oil) and emulsified in natural ox bile, caused a distinct blue fluorescence in the most superficial layer of the glandular mucosa. The same phenomenon was observed when lecithin was used.

Emulsions of light petrolatum are, in contrast to plain light petrolatum, able to penetrate the most superficial layer of the glandular mucosa. However, the blue fluorescence disappeared rapidly in these cases.

In general, the fluorescence intensity was greatest at the mucous surface, becoming gradually weaker deeper in the stomach wall (cf. Fig. 2). BP seemed to penetrate comparatively rapidly to a certain depth which is characteristic of each vehicle; after about 30 minutes the depth of penetration increased only insignificantly, indicating that further transport of the fat-soluble BP is slow. The depth of the penetration did not depend on the actual thickness of the glandular mucosa; in regions in which the thickness of the wall was less, all layers of it had taken up blue fluorescent material, while in the thicker regions of the same stomach only the equal superficial layers were fluorescent. In several cases the wall of the pyloric region was thinner than that of the others. In cases in which the whole mucosa had absorbed BP, there appeared to be a well developed network in the muscle layers (24). A similar structure existed in the corresponding part of the forestomach as well (Figs. 3 and 4). There were great differences in penetration power among various solvents (25).

The nuclei were free of fluorescent material. There were no noticeable differences in the intracytoplasmic distribution and localization of BP in various cell types; intracytoplasmic fluorescent granules were not noted (Fig. 5).

A strong mucigenic action, most probably of a local nature, was observed in connection with sodium cholate, sodium taurocholate, and sodium myristyl sulfate. Some of the earlier authors have explained that the limited gastric absorption may partly depend upon the so-called washing effect of the gastric secretion. However, a strong secretion, induced artificially by histamine administration, weakened the penetration power of the vehicles only slightly.

When BP, solubilized in aqueous solution, was given as a single application, the blue fluorescence disappeared from the glandular mucosa in about 30–45 minutes; but when given in anhydrous lipohydrophilic substances with a strong penetration ability (e.g., polyethylene glycols, Tritons), the time of disappearance was 6 hours.

Forestomach.—BP, dissolved in any of the vehicles studied, easily penetrated the wall of the mouse forestomach (Figs. 2, *A–D*, 3, and 4), as

previously described (24). Similar observations were made on the esophagus and the ridge between the forestomach and the glandular stomach (Fig. 4), both of which are also lined with squamous epithelium. In general, the blue fluorescence disappeared from the forestomach wall, in 24–48 hours. All the results are compatible with the observations made in feeding experiments in which tumors developed only in the forestomach (1, 4, 23, 28).

Stomach of the Cat

Experiments were made on cats, because the cat's stomach rather closely resembles the human stomach both anatomically and physiologically.

The results were essentially similar to those obtained in experiments with mice (Figs. 6 and 7).

Intestine of Mice and Cats

Absorption of the blue fluorescent material from the lumen of the intestine was, in general, similar in mice and cats. BP dissolved in various fats, emulsions, and in the simultaneously lipo- and hydrophilic substances easily penetrated the tissues of this segment of the alimentary canal. A very strong BP fluorescence was seen intracytoplasmically in the structures of the wall of the small intestine (Figs. 8 and 9) and in that of the large intestine.

Differences in penetration power among the various types of vehicles for BP corresponded to well known physiologic principles.

Thoracic Duct of Mice and Cats

After instillation of BP into the gastrointestinal tract, blue fluorescent material was seen in the contents of the mesenterial lymphatics, and in lymph of the thoracic duct in connection with all types of vehicles used.

In addition, we have reported in another connection that at least a considerable part of instilled BP is carried further by the chylomicrons of the blood (26). As is known, the water-insoluble alimentary fats in the blood are transported as chylomicrons, the composition of which varies depending on several factors (9).

DISCUSSION

Gastric absorption was studied earlier chiefly by ligating the pylorus and, after a variable period, by withdrawing samples of stomach contents and of blood. However, in experiments on mice, the histologic technic may be the most suitable one, because operative procedures change the physiologic conditions in the stomach. In addition, the histologic method has the advantage of following the absorption directly in the stomach wall, which is the actual problem. The word "absorption" has here been used in its broadest sense, including passive penetration. Although the limits of the sensitivity of fluorescence microscopy as used in the present work are not fully known, it is certain that this technic is considerably more accurate than those previously used.

As recorded in Fig. 2 *D*, BP dissolved in plain fats or in light petrolatum did not cause any visible blue fluorescence in the glandular mucosa. This is understandable, because little or no fat is absorbed by the stomach wall under normal conditions (cf. 3, 14, 19).

First, after being emptied into the small intestine, alimentary fat comes in contact with the bile, one of the most important factors in fat absorption. The sodium salts of the bile acids are able to make fat-soluble substances (18, 32), including carcinogenic hydrocarbons (8), water-soluble. According to the lipolytic theory of fat absorption, fat, before absorption, is enzymatically hydrolyzed into split products, which are water-soluble. However, according to the fat partition theory (10), a certain amount of fat, associated with bile salts and some hydrolytic products, is absorbed directly in insoluble form; the size of the fat particles must be less than about 0.5 μ (cf. 9).

However, conditions in the intestine and in the unchanged stomach are not comparable—practically no emulsifying or lipolytic agents occur in normal stomachs. Under certain pathologic conditions, e.g., hypochlorhydria or achlorhydria, gastric absorption of fat-soluble substances would, however, be possible. Though it is unknown how often regurgitation of the contents of the duodenum into the stomach occurs in the so-called precarcinogenic period of gastric carcinogenesis, it has been considered a possible contributory agent in the genesis of gastric cancer. In addition, Ivy (13) observed that the pyloric mucosa and sometimes the mucosa along the lesser curvature—i.e., in those regions in which about two-thirds of gastric carcinomas occur—was stained yellow with bile pigment when the contents of the duodenum were present in the stomach.

It appeared in the present work that BP, dissolved in fats and emulsified in natural bile, as well as solubilized by some bile salts or in synthetic bile, caused a distinct penetration into the superficial layer of the wall of the glandular stomach. Furthermore, BP, solubilized both in aqueous and in nonaqueous synthetic vehicles, possessing simultaneously lipo- and hydrophilic properties (cf. 24, 25), is easily absorbed by the glandular mucosa of the normal stomach in mice and cats. All the substances mentioned above have a

common characteristic: *they are able to bring the fat-soluble carcinogenic hydrocarbons into aqueous solution.* The physico-chemical properties of the vehicles used are reported in the following paper (7). There is no real basis for the postulated "mucous barrier" to absorption of carcinogenic hydrocarbons in the stomach.

It is not sufficient that the carcinogens are absorbed by the stomach wall. The agents must remain *in situ* for some time before tumors develop (cf. 11). However, it was noted in the present investigation that the blue fluorescence of the ingested material disappeared from the normal glandular mucosa during a time which evidently is too short to induce adenocarcinomas of the stomach. It seems plausible, however, that gastric adenocarcinoma could develop in a stomach which is pathologically altered, since this would provide an entry for some fat-soluble carcinogens (e.g., "heated lipids"). Thus, though the significance of chronic atrophic gastritis in gastric carcinogenesis has not been established with certainty, it may be said that a great bulk of gastric carcinomas in man arises from gastritis. Because the intestinal wall, physiologically, and the coat of the normal stomach in the presence of certain solvents absorb the fat-soluble carcinogen (cf. Figs. 5, 6, and 8), it can be expected a priori that the altered, newly formed coat of the gastritic stomach will also resemble the actual intestinal lining when factors participating in the absorption of fat-soluble substances are present. In addition, it is understandable that owing to the alterations in the structure of the wall of the gastritic stomach (together with the general weakness of the lacteal system in it) the carcinogen could not be further transported as easily as it could following absorption by the intestine. It may be that gastric adenocarcinoma occurs in a stomach in which there is a "functional and anatomic intestinalizatıon." Certain clinical observations strongly support this assumption.

Investigations, planned along the principles presented above, are now in progress.

SUMMARY

Gastric absorption of 3,4-benzpyrene in mice and cats was investigated following gastric instillation of a single dose of the hydrocarbon, taking into account the physiologic knowledge of absorption of ingested material in different parts of the gastrointestinal tract. The rate of absorption was determined directly in the wall of the normal stomach with fluorescence microscopy.

No signs of absorption by the gastric glandular mucosa were observed after administration of the hydrocarbon in natural fats or in liquid petrolatum. These results are in agreement with the observations made in feeding experiments in which adenocarcinomas were not produced when fats were used as solvents for the carcinogen.

On the contrary, benzpyrene was able to penetrate into the glandular mucosa of the stomach when dissolved in fats or in light petrolatum and emulsified in natural bile or solubilized by some bile constituents (both natural and synthetic ones).

A strong absorption occurs when synthetic substances possessing simultaneously certain lipo- and hydrophilic characteristics, with the aid of which carcinogenic hydrocarbons are brought into aqueous solutions, are used as vehicles for the hydrocarbon. It is of great significance that these substances have certain common properties with bile. However, the blue fluorescence disappears comparatively rapidly from the glandular mucosa of the normal stomach.

The results have been discussed with particular reference to some physiologic facts and pathologic alterations in the stomach.

REFERENCES

1. Armstrong, E. C., and Bonser, G. M. Squamous Carcinoma of the Forestomach and Other Lesions in Mice Following Oral Administration of 3:4:5:6-Dibenzcarbazole. Brit. J. Cancer, **4**:203–11, 1950.
2. Barrett, M. K. Avenues of Approach to the Gastric-Cancer Problem. J. Nat. Cancer Inst., **7**:127–57, 1946.
3. Bloor, W. R. Biochemistry of the Fatty Acids. New York: Reinhold Publishing Corp., 1943.
4. Collins, V. J.; Gardner, W. U.; and Strong, L. C. Experimental Gastric Tumors in Mice. Cancer Research, **3**:29–35, 1943.
5. Denton, R. W.; Sheldon, P.; and Ivy, A. C. Attempts To Produce Gastric Carcinoma Experimentally in a Gastric Ulcer. Cancer Research, **10**:684–85, 1950.
6. Domagk, G. Weitere experimentelle Untersuchungen über die Ursachen des Krebses. Ztschr. f. Krebsforsch., **48**:288–97, 1939.
7. Ekwall, P.; Ermala, P.; Setälä, K.; and Sjöblom, L. Gastric Absorption of 3,4-Benzpyrene. II. Significance of the Solvent for the Penetration of 3,4-Benzpyrene into the Stomach Wall. Cancer Research, **11**:758–63, 1951.
8. Ekwall, P., and Setälä, K. Solutions of Carcinogenic Hydrocarbons in Solvents of Both Lipophilic and Hydrophilic Character. Acta de l'union internationale contre le cancer, **7**:120–25, 1950.
9. Ermala, P. On the Postprandial Chylomicronemia. Diss. Acta Physiol. Scandinav. (in press).
10. Frazer, A. C. The Absorption of Triglyceride Fat from the Intestine. Physiol. Rev., **26**:103–12, 1946.
11. Hollander, F.; Sonnenblick, B. P.; and Sober, H. Experimental Impairment of the Gastric Mucous Barrier in Dogs. J. Nat. Cancer Inst., **7**:361–64, 1947.
12. Hoves, E. L., and Oliveira, J. R. Early Changes in the Experimentally Produced Adenomas and Adenocarcinomas of the Stomach. Cancer Research, **8**:419–24, 1948.
13. Ivy, A. C. Gastric Physiology in Relation to Gastric Cancer. J. Nat. Cancer Inst., **5**:313–37, 1945.

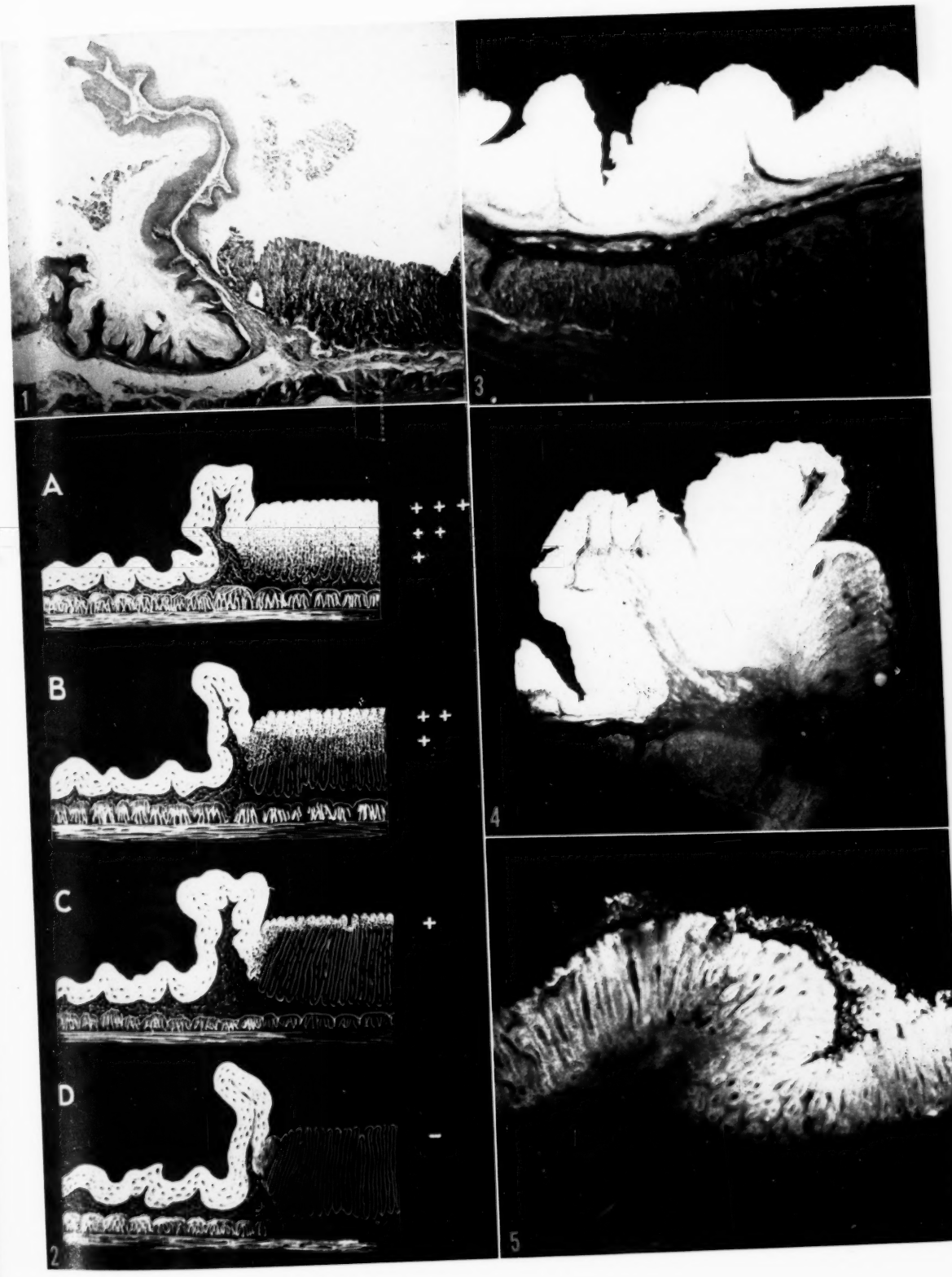
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FIG. 1.—Section of the mouse stomach: left, forestomach lined with squamous epithelium; right, glandular stomach—separated from one another by the ridge composed of squamous epithelium. (Hyperkeratotic changes caused by longer carcinogen treatment in this case.) H. van G. ×30.

FIG. 2, *A–D*.—Diagrammatic representation of the wall of the mouse stomach, illustrating the fluorescence intensity and the penetration ability 30 min. after gastric instillation of BP in various vehicles. *A*, Penetration depth about 0.45/0.60 mm (e.g., anhydrous polyethylene glycols, Tritons) (cf. Figs. 3–5). *B*, Penetration depth about 0.15–0.30/0.60 mm. *C*, Penetration depth about 0.15/0.60 mm (e.g., fat constituents, or lecithin). *D*, No visible blue fluorescence in the glandular mucosa (natural fats, light petrolatum).

FIG. 3.—(Figs. 3–6 and 8 are unstained frozen sections photographed in UV light 30 min. after gastric instillation of BP.)

Lipo-hydrophilic Carbowax 1,500. The keratinized layers as well as the stratified epithelium of the mouse forestomach are strongly fluorescent with a dazzling white tinge; in the muscle layer is a network containing blue fluorescent material. ×110.

FIG. 4.—The ridge of the mouse stomach. Anhydrous Triton. ×110.

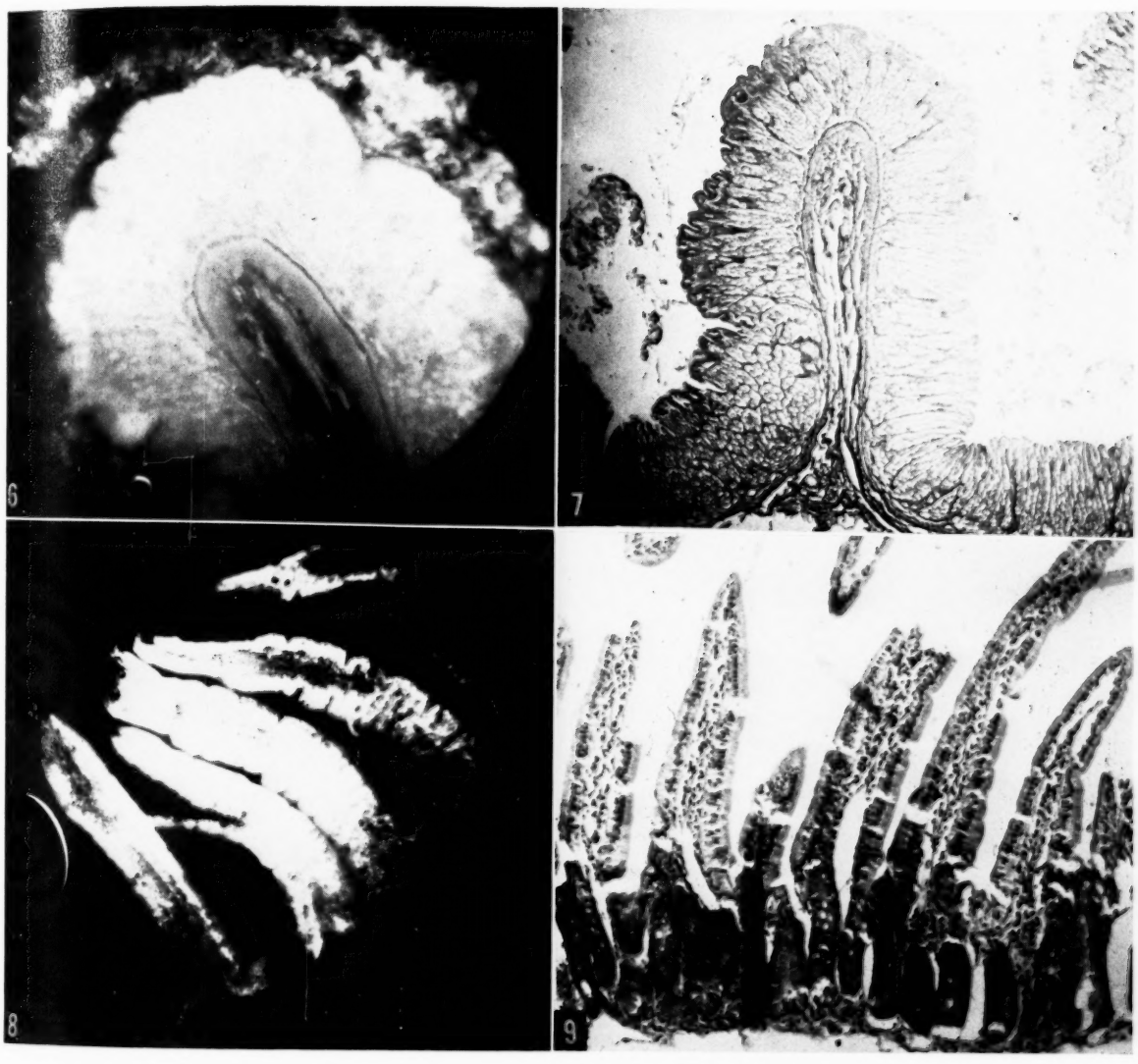
FIG. 5.—Glandular mucosa of the mouse stomach. Lipo-hydrophilic polyethylene glycol 200. The gland cells have taken up blue fluorescent material. Nuclei contain no fluorescent material. Mucus has also taken up fluorescent material. ×110 (cf. especially Fig. 8).

FIG. 6.—Cat stomach. Lipo-hydrophilic Carbowax 1,500. White fluorescence in all layers of the glandular mucosa. Mucus has also taken up fluorescent material. ×110 (cf. Figs. 5 and 8).

FIG. 7.—Cat stomach. Gomori stain. ×75.

FIG. 8.—Small intestine of the cat. Arachidis oil. The cells of the lining of the intestinal villi have taken up strongly (blue) fluorescent material. Nuclei contain no fluorescent material. ×120. This finding fully corresponds to the physiologic absorption mechanism in the small intestine (cf. Figs. 5 and 6).

FIG. 9.—Small intestine of the cat. Gomori stain. ×120.


6
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9

14. KAREL, L. Gastric Absorption. Physiol. Rev., **28**:433–50, 1948.
15. LANE, A.; BLICKENSTOFF, D.; and IVY, A. C. The Carcinogenicity of Fat "Browned" by Heating. Cancer, **3**:1044–51, 1950.
16. LARIONOW, L. T. On the Fate of Carcinogenic Hydrocarbons in the Animal Body. Cancer Research, **7**:230–40, 1947.
17. LORENZ, E. Preparation of Emulsions and Suspensions Containing Carcinogenic Hydrocarbons. J. Nat. Cancer Inst., **10**:355–58, 1949.
18. MCBAIN, J. W.; MERRILL, R. C.; and VINOGRAD, J. R. The Solubilization of Water-insoluble Dye in Dilute Solutions of Aqueous Detergents. J. Am. Chem. Soc., **63**:670–76, 1941.
19. PETERS, J. P., and VAN SLYKE, D. D. Quantitative Clinical Chemistry, Vol. **1**. 2d ed. Baltimore: Williams & Wilkins Co., 1946.
20. ROFFO, A. H. Producción de úlceras y tumores malignos en el aparato digestivo por la ingestión de alimentos con colesterina irradiada. Bol. Inst. de med. exper. para el estud. y trat. d. cáncer, **15**:407–521, 1938.
21. ———. Pirólisis del colesterol; alquitrán cancerigeno del colesterol. Bol. Inst. de med. exper. para el estud. y trat. d. cáncer, **18**:929–48, 1941.
22. RUSCH, H. P.; BAUMANN, C. A.; and MAISON, G. L. Production of Internal Tumors with Chemical Carcinogens. Arch. Path., **29**:8–19, 1940.
23. SAXÉN, E.; EKWALL, P.; and SETÄLÄ, K. Squamous-Cell Carcinoma of the Forestomach in Mice, Following Oral Administration (Cannula Feeding) of 9:10-Dimethyl-1:2-Benzanthracene Solubilized in Aqueous Solution of an Association Colloid. Acta path. et microbiol. Scandinav., **27**:270–75, 1950.
24. SETÄLÄ, K., and EKWALL, P. Penetration of Benzpyrene into the Stomach Wall of Mouse. Science, **112**:229–31, 1950.
25. ———. Some Observations on Chemical Carcinogenesis Using New Types of Solvents for the Carcinogenic Hydrocarbons. Acta de l'union internationale contre le cancer, **7**:160–66, 1950. Int. Cancer Congress, July, 1950, Paris.
26. SETÄLÄ, K., and ERMALA, P. Chylomicrons as Carriers for Carcinogenic Hydrocarbons. Science (in press).
27. SETÄLÄ, K.; HOLSTI, L. R.; and ERMALA, P. Absorption of the Fluorescent Constituents of the Tobacco Tar (in press).
28. STEWART, H. L., and LORENZ, E. Tumors of Alimentary Tract in Mice Fed Carcinogenic Hydrocarbons in Mineral-Oil Emulsions. J. Nat. Cancer Inst., **9**:173–80, 1948.
29. STEWART, H. L.; HARE, W. V.; LORENZ, E.; and BENNETT, J. G. Adenocarcinoma and Other Lesions of the Glandular Stomach of Mice, Following Intramural Injection of 20-Methylcholanthrene. J. Nat. Cancer Inst., **10**:359–60, 1949.
30. VAN PROHASKA, J.; BRUNSCHWIG, A.; and WILSON, H. Oral Administration of Methylcholanthrene to Mice. Ann. Surg., **38**:328–33, 1939.
31. WATERMAN, N. Experimental Production of Carcinoma in the Stomach of Mice. Acta Cancrologia, **2**:375–88, 1936.
32. WIELAND, H., and SORGE, H. Untersuchungen über die Gallensäuren. Ztschr. f. physiol. Chem., **97**:1–27, 1916.

Gastric Absorption of 3,4-Benzpyrene

II. The Significance of the Solvent for the Penetration of 3,4-Benzpyrene into the Stomach Wall

PER EKWALL, PENTTI ERMALA, KAI SETÄLÄ, AND LARS SJÖBLOM

(Institute of Physical Chemistry, Åbo Akademi, Åbo, and the Departments of Anatomy and Roentgenology, Second Medical Clinic, University of Helsinki, Helsingfors, Finland)

We have systematically studied the significance of the nature of the solvent for the penetration of 3,4-benzpyrene into the stomach wall. Efforts were particularly made to elucidate the significance of the lipophilic and hydrophilic properties of the solvent and of the balance between these properties.

In these studies a large number of solvents were employed for the administration of 3,4-benzpyrene. The solutions of the hydrocarbon were instilled into the stomachs of mice and cats and the rate of the absorption of the fluorescent carcinogen determined directly in the wall of the stomach in the manner described in the first paper of this series (7). The intensity of fluorescence in the wall of the glandular stomach was estimated by using an arbitrary gradation (Fig. 2, preceding paper).

EXPERIMENTAL RESULTS

Stomach of the Mouse

Anhydrous solvents.—Table 1 contains the results of experiments in which nine anhydrous solvents of different chemical composition were employed.

Forestomach.—When administered in the solvents given in Table 1, benzpyrene easily penetrated the wall of the mouse forestomach. The results indicate that the type and properties of the solvent in which BP is dissolved have no influence on the penetration into the wall of the forestomach. The results given in Tables 2–4 confirm this opinion and show that BP also penetrates into the forestomach when solubilized in aqueous solutions of association colloids or when dissolved in water-oil emulsions.

Glandular stomach.—The properties of the solvent are, however, decisive for the penetration of the hydrocarbon into the walls of the glandular portion of the stomach. With respect to the ability of the solvent to bring about penetration, one can divide the solvents into three groups.

a) Solvent group 1: After the feeding of BP dissolved in solvents of this group, no blue fluorescence was observed in the glandular wall. To this group belong markedly lipophilic substances such as paraffin oil and the triglycerides of fatty acids (Fig. 2 *D*, preceding paper).

b) Solvent group 2: Following the feeding of BP dissolved in solvents of this group, a fluorescence was observed only in the outermost layer of the glandular mucosa (Fig. 2 *C*, preceding paper). The substances belonging to this group, oleic acid and oleyl alcohol, are mainly lipophilic but also contain clearly hydrophilic groups.

c) Solvent group 3: The feeding of BP dissolved in solvents of this group resulted in a penetration of the fluorescent material not only into the superficial layers but also to a greater depth into the wall of the glandular mucosa (Fig. 2 *A*, 2 *B*, preceding paper). The main representatives of this group are the polyethylene glycols (Carbide and Carbon Chemicals Corp., New York, N.Y.) and the association colloids (the nonionic alkyl aryl polyether alcohol "Triton N," the anionic sulfonic acid compound "Triton 720," Rohm and Haas Co., Philadelphia, Pa., and the cationic alkyl dimethyl benzyl ammonium chloride "Rodalon," Rhodas Chemical Corp.). (These commercial products were used as purchased.)

The results show that a solvent able to effect the penetration of BP into the glandular mucosa must possess, besides a lipophilic part, one or more hydrophilic groups of adequate strength. A marked penetration is observed only when the hydrophilic properties of the solvent predominate over the lipophilic properties. To obtain further proof for this assumption, we conducted experiments with the solvents mentioned in Table 2. The balance between the hydrophilic and lipophilic properties of these six nonionic compounds

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(commercial products from Atlas Powder Co., Wilmington, Del.) varies greatly. This balance is designated by a number in column 2: the lower the value of this HLB index[1] (hydrophilic-lipophilic-balance), the more dominant is the lipophilic character; the closer it approaches the value 20, the more the balance between the hydrophilic and lipophilic properties has shifted in favor of the former.

Sorbitan trioleate (HLB 1.8), which is the most lipophilic member in the series, is not able to promote the penetration of BP into the wall of the glandular stomach. It thus belongs to solvent group 1. Sorbitan monolaurate (HLB 8.6) and polyoxyethylene sorbitan trioleate (HLB 11.0) induce a weak penetration of BP and must hence be included in group 2. Polyoxyethylene sorbitan monolaurate (HLB 16.7), polyoxyethylene sorbitan monopalmitate (HLB 15.6), and polyoxyethylene stearate (HLB 17.9) effect a strong penetration and hence belong to solvent group 3. The latter three substances are typical nonionic association colloids.

Aqueous solutions as solvents for BP.—Table 3 indicates how far the solvents of group 3 can maintain their power of promoting the penetration of

[1] "Atlas Surface Active Agents," Atlas Powder Company, Wilmington, 1948.

TABLE 1

FLUORESCENCE IN THE STOMACH WALL OF MICE 30 MINUTES AFTER GASTRIC INSTILLATION OF BP IN VARIOUS ANHYDROUS SOLVENTS

Group	Solvent	Conc. of BP (per cent)	Forestomach	Glandular mucosa: Depth of penetration estimated from the mucous surface (mm.) 0.15	0.30	0.45	0.60
1	Liquid petrolatum	0.5	++++	−	−	−	−
	Arachidis oil	0.5	++++	−	−	−	−
2	Oleyl alcohol	0.5	+++	(+)	−	−	−
	Oleic acid	0.5	++++	(+)	−	−	−
3	Alkyl dimethyl benzyl ammonium chloride, "Rodalon"	0.5	+++	+(+)	(+)	−	−
	Sulfonated polyether compound, "Triton 720"	0.5	++++	+++	++	+	−
	Alkyl aryl polyether alcohol, "Triton N"	0.5	++++	+++	++	+	−
	Polyethylene glycol, "200"	0.5	++++	+++	++	+	−
	Polyethylene glycol, "Carbowax 1500"	0.5	++++	+++	++	+	−

TABLE 2

INFLUENCE OF THE BALANCE BETWEEN THE HYDROPHILIC AND LIPOPHILIC PROPERTIES OF THE SOLVENT ON THE FLUORESCENCE IN THE STOMACH WALL OF MICE 30 MIN. AFTER GASTRIC INSTILLATION OF BP SOLUTION

Group	Solvent	HLB* index	Conc. of BP (per cent)	Forestomach	Glandular mucosa: Depth of penetration estimated from the mucous surface (mm.) 0.15	0.30	0.45	0.60
1	Sorbitan trioleate	1.8	0.5	++	−	−	−	−
2	Sorbitan monolaurate	8.6	0.5	+++	(+)	−	−	−
	Polyoxyethylene sorbitan trioleate	11.0	0.5	+++	+	−	−	−
3	Polyoxyethylene sorbitan monopalmitate	15.6	0.5		+++	++	(+)	−
	Polyoxyethylene sorbitan monolaurate	16.7	0.5	+++	+++	+	−	−
	Polyoxyethylene stearate	17.9	0.5		+++	++	+	−

* Solvents with low HLB index are predominantly lipophilic in character, the substances with a higher index being more hydrophilic.

TABLE 3

INFLUENCE OF THE CONCENTRATION OF THE HYDROLIPOPHILIC SOLVENT ON THE FLUORESCENCE IN THE STOMACH WALL OF MICE 30 MIN. AFTER GASTRIC INSTILLATION OF BP SOLUTION

"Triton N"* concentration in aqueous solution (per cent)	Conc. of BP (per cent)	Forestomach	Glandular mucosa: Depth of penetration estimated from the mucous surface (mm.) 0.15	0.30	0.45	0.60
100	0.1	++	+	−	−	−
90	0.1	+++	+	−	−	−
80	0.1	+++	+	(+)	−	−
60	0.1	+++	+	(+)	−	−
30	0.1	+++	+(+)	(+)	−	−
20	0.1	+++	+(+)	+	−	−

* A nonionic association colloid.

BP into the glandular mucosa of the stomach upon dilution with water.

With a constant BP content of 0.1 per cent, the ability of the solvent to promote the penetration of the carcinogen increases as the colloid concentration is decreased from 100 per cent to 20 per cent by the addition of water. At least in the more dilute solutions of this series we are dealing with solutions containing micelles in which the hydrocarbon is solubilized (5, 6). It was found in other experiments that the degree of dilution is of no great importance when the BP concentration is high.

TABLE 4

FLUORESCENCE IN THE STOMACH WALL OF MICE 30 MIN. AFTER GASTRIC INSTILLATION OF BP SOLUBILIZED IN AQUEOUS ASSOCIATION COLLOID SOLUTIONS OR DISSOLVED IN EMULSIONS

	Colloid conc. (per cent)	Conc. of BP (per cent)	Forestomach	Glandular mucosa Depth of penetration estimated from mucous surface (mm.) 0.15	0.30
A. Aqueous solution of:					
"Triton N"	6.4	0.0215	+++	(+)	–
Cetyl trimethyl ammonium bromide	5	0.0215	+++	(+)	–
Sodium myristyl sulfate	20	0.04	+++	+	–
Sodium taurocholate	70	0.06	+++	+	–
Sodium taurocholate	25	0.02	++	(+)	–
"Synthetic bile," (sodium taurocholate, 9.3 per cent; sodium oleate, 1.2 per cent; lecithin, 0.22 per cent; cholesterol, 0.034 per cent)	about 10	0.01	+++	(+)	–
Ox bile		<0.01	++	–	–
B. Aqueous emulsions of:					
Arachidis oil in ox bile	aa	0.25	+++	(+)	–
Light mineral oil in water containing sodium taurocholate, glyceryl monostearate, cetyl alcohol, pH 9 (according to Lorenz)	0.8:9	0.1	+++	(+)	–
Lecithin in water	aa	0.5	+++	+	–

Table 4 *A* contains some results obtained with aqueous solutions of different association colloids. They are not in all cases strictly comparable, since the BP concentration could not be kept constant because the solutions differed greatly in their power to solubilize the hydrocarbon. Of particular interest are the experiments with solutions of bile salts and with bile. In all these a fluorescence was observed only in the superficial layer of the stomach wall. The reason for this weak fluorescence may be the low BP concentration, particularly in the experiment with ox bile.

Table 4 *B* contains the results of experiments conducted with BP dissolved in the droplets of oils or lecithin emulsified in certain aqueous solutions.

The significance of the BP concentration.—Experiments with aqueous solutions containing low concentrations of BP raised the question of how much the concentrations of BP can be reduced until the penetration of carcinogen is no longer observed with the experimental technic employed. Although this has not yet been investigated, we have been able to make a number of observations in special cases which elucidate this question.

TABLE 5

THE INFLUENCE OF BP CONCENTRATION ON THE BLUE FLUORESCENCE IN THE GLANDULAR MUCOSA OF MICE 30 MIN. AFTER GASTRIC INSTILLATION OF BP DISSOLVED IN ANHYDROUS "TRITON N"

Conc. of BP (per cent)	Glandular mucosa Depth of penetration estimated from the mucous surface (mm.) 0.15	0.30	0.45	0.60
0.5	+++	++	+	–
0.4	++	+	–	–
0.3	+	+	–	–
0.2	+	–	–	–
0.1	+	–	–	–

As expected, these experiments show that the fluorescence of the tissues decreases both in the depth to which it extends and in intensity as the BP concentration in the solution is diminished. The lowest limit for an observable fluorescence varies greatly with the colloid concentration. Whereas with 100 per cent "Triton N" this limit is somewhat lower than 0.1 per cent, in 6.4 per cent solutions (Table 4 *A*) it is approximately 0.02 per cent. From this it appears that with our method of

observation the lower limit is determined not only by the BP concentration but also by the state and type of the solvent employed. Many factors may contribute to this result. The fluorescence of BP differs in different solvents. The powers of the solvents to keep BP dissolved on dilution are very different (5, 6). The elimination of BP from the cells, by transport or in some other way, may depend on the solvent. We believe that, if the solvent possesses hydrophilic-lipophilic properties in the ratio characteristic of the substances of group 3 and is able to keep BP in solution under the conditions prevailing in the stomach, the fact that no fluorescence is observed in the glandular mucosa at low BP concentrations does not necessarily mean a sudden decrease in the power of the solvent to promote the penetration. It seems more likely that a penetration of BP actually occurs but is not observable. This probably occurs with the dilute aqueous solutions of many of the association colloids.

Stomach of the Cat

BP, dissolved in paraffin oil, arachidis oil, anhydrous polyethylene glycol (Carbowax 1500), or in an aqueous solution containing 20 per cent sodium myristyl sulfate, was introduced into four cats by stomach tube. The lipophilic solvents of group 1 are not able to effect a penetration of the fluorescent substance into the glandular mucosa of the stomach. "Carbowax 1500" and the sodium myristyl sulfate solution induced a strong penetration in all parts of the stomach.

DISCUSSION

The substances of solvent group 3, which effect a strong penetration of BP into the glandular mucosa of the stomach, are organic compounds containing one or several markedly lipophilic hydrocarbon groups and one or more radicals which are definitely hydrophilic. In all these compounds the balance between these properties is toward the hydrophilic side. The lipophilic properties of the solvents make it possible for the anhydrous substances to dissolve the carcinogen. Owing to their hydrophilic properties, they are miscible with water. Because of the combination of lipophilic and hydrophilic properties the carcinogen remains dissolved, even though the solution is diluted with water.

In another connection (1,2,5,6) we have already considered the physico-chemical properties of these solvents and the solutions obtained when carcinogens are dissolved in them. Here, we only wish to emphasize some of the properties which have a direct bearing on the ability of the solvents to promote the penetration of the carcinogen into the stomach wall.

The two main types of substances in group 3, the polyethylene glycols and the association colloids (soaps and soaplike compounds), differ in their behavior when diluted with water. The power of the former to keep carcinogenic hydrocarbons dissolved rapidly diminishes when water is added. The lowest concentrations at which the polyethylene glycols are still able to dissolve carcinogenic hydrocarbons are in the neighborhood of 4–5 per cent. But even in a 50 per cent aqueous solution of "Carbowax 1500," the solubility of 20-methylcholanthrene, for example, has decreased to 0.0005 per cent. Thus, when a polyethylene glycol containing 0.5 per cent BP is diluted with water, the greater part of the carcinogen is precipitated. This explains why BP crystals are often observed on the surface of the mucosa when solvents of this type are employed. It may also be expected that the carcinogen will be precipitated in the tissues as a result of dilution by the water present.

The situation is quite different in the case of the association colloids. On dilution with water they form micellar solutions. As is well known, the molecules or ions of the typical association colloids associate in aqueous solution to give large aggregates, micelles, in which the molecules and ions are arranged so that their hydrocarbon parts are situated beside one another in the inner parts, while the hydrophilic groups are oriented outward against the water. These micelles solubilize the carcinogens and hold them in solution. These aqueous carcinogen solutions are clear and stable and not suspensions or emulsions (4–6). When the colloid concentration is reduced below the so-called critical concentration the micelles are broken up, and the solutions are no longer able to solubilize the carcinogen. This critical concentration, however, is usually very low, between 0.002 and 1 per cent. Even at fairly high dilutions, the association colloids are able to keep the carcinogen solubilized (2 per cent solutions of "Triton N" and sodium myristyl sulfate, for example, dissolve approximately 0.012 and 0.005 per cent BP, respectively). Owing to the high solubilizing power of these solutions, no carcinogen crystals, in general, were observed on the surface of the mucosa in the experiments in which they were used. Provided that the micelles are stable in the environment in question, there may be a lesser tendency for the carcinogen to precipitate in the tissues, and the probability of its elimination by being carried away may be greater.

In our experiments we have employed repre-

sentatives of the three main types of association colloids: the cationic (with positively charged micelles), the anionic (with negatively charged micelles), and the nonionic (with electrically neutral micelles). Regardless of the type and charge of the micelles, both in the anhydrous form and in aqueous solution, the association colloids promote the penetration of the dissolved carcinogen into the glandular mucosa of the stomach.

The anionic association colloids in which the hydrophilic properties are due to an anionic carboxyl group ($-COO^-$) are unstable in acid solution. The anionic association colloids containing sulfate or sulfonate hydrophilic groups, on the other hand, form micelles that are stable in acid media. They maintain their power to solubilize carcinogens even under the acid conditions prevailing in the stomach. This is true, for example, with solutions containing alkyl sulfates and taurocholate (3). Cationic association colloids form micelles that are stable in acid solution (some of them are unstable in an alkaline environment). The micelles of the nonionic association colloids are stable both in acid and alkaline solutions.

A characteristic of aqueous solutions of the association colloids is their high surface activity; the molecules and ions of the colloids concentrate at the interfaces. Because of this they have low surface tensions and low interfacial tensions against lipophilic media. They spread easily over both lipophilic and hydrophilic surfaces. Similarly, aqueous solutions of the polethylene glycols have relatively low surface tensions and good spreading properties.

It is believed that the mucin layer which covers the whole glandular mucosa usually prevents the carcinogen from coming into direct contact with the stomach wall. Since a direct contact is the first condition for the absorption of the carcinogenic hydrocarbon, it is obvious that the solvents of group 3 possess properties that make such a contact possible.

From the physico-chemical point of view it is clear that the lipophilic, water-insoluble solvents of group 1 are not able to displace the hydrophilic mucin from the predominantly hydrophilic glandular mucosa. Similarly, it may be supposed that the possibilities for this will increase with the hydrophilic character of the solvent. To what extent the removal of the mucin involves direct solution, emulsification, or precipitation of this substance or surface-chemical displacement is still difficult to envisage.

On the other hand, it is not quite clear whether a direct contact between the glandular mucosa and the carcinogen solution is a sufficient physico-chemical condition for absorption. When contact is established, the nature of the solvent and the state of the dissolved carcinogen may be of importance. In all cases where we have observed a strong absorption, the solvent was miscible with water or was itself an aqueous solution. It seems that the carcinogen penetrates into the mucosa along with the solvent, either molecularly dissolved in the solvent or solubilized in the micelles.

Whether the carcinogen is also able to penetrate into the mucosa when dissolved in emulsified particles of fats and other predominantly lipophilic substances is not yet clear. Our experiments with emulsions may be construed as evidence for such a penetration, but this is not certain, since these emulsions also contained BP solubilized in the micelles of the aqueous phase. Thus, the penetration in these cases can also take place from the aqueous solution and its micelles.

It is not known whether the weak penetration observed with the water-insoluble solvents of group 2 occurs by a direct transfer of only the carcinogen from the solvent to the tissues or by some other mechanism.

Some of the substances whose ability to promote penetration into the glandular mucosa of the stomach has been studied are often present in natural food: fatty acid glycerides, lecithin, and fatty acids. The latter two belong to group 2 and according to our experiments possess a certain, although very slight, ability to promote the penetration of BP. Substances of group 3 which possess great penetration-promoting properties are seldom found in ordinary food.

An association colloid solution capable of solubilizing carcinogenic hydrocarbons, however, enters the stomach when bile is regurgitated from the intestine. The micelles in bile are evidently mixed micelles consisting of taurocholate, glycocholate, and fatty acid anions, and probably also contain some cholesterol and possibly other lipids as well. These micelles are to some extent stable and can thus solubilize carcinogens in acid environment (3). We did not, however, observe a fluorescence in the glandular mucosa after feeding BP solubilized in natural ox bile. This negative result may be due to the extremely low BP concentration (<0.01 per cent). When a "synthetic bile" containing sodium taurocholate, sodium oleate, cholesterol, and lecithin was used as solvent for BP (0.01 per cent BP), a weak but distinct blue fluorescence could be observed in the superficial layer of the glandular mucosa. An emulsion of arachidis oil in ox bile (equal parts, 0.25 per cent BP) gave a slightly stronger fluorescence. When a concentrated taurocholate solution containing 0.06 per

cent BP was fed to the experimental animals, the fluorescence was distinct. We thus conclude that when bile is regurgitated into the stomach, conditions may prevail, especially if the pH in the stomach is higher than usual, which make possible the penetration into the glandular mucosa of carcinogens which have entered the stomach along with food or have accumulated in the bile.

The hydrophilic-lipophilic substances of group 3 may, however, enter the stomach more or less accidentally. The possibilities for this have increased since such substances have come into use in ever increasing quantities in food and pharmaceutical products. For example, certain polyoxyethylene sorbitan esters of fatty acids ("Tweens") are added to bread and similar products.

All these substances have very low critical concentrations and are thus able to solubilize carcinogenic hydrocarbons even in extremely high dilutions. Our investigation has not proved that highly diluted aqueous solutions of association colloids are able to promote the penetration of solubilized carcinogens; from a physico-chemical point of view, however, there is no reason to believe that this ability is lost before the concentration of the colloid is reduced to the dilution at which the micelles disappear.

If it could be shown that fat can be absorbed in the stomach as emulsified in fine particles, there would be every reason to believe that the natural emulsifiers and the synthetic emulsifiers included in our solvent group 2 would possess a still greater ability to effect the penetration of carcinogens into the glandular mucosa than is evident from the investigations we have conducted up to the present.

SUMMARY

The ability of various solvents to effect the penetration of 3,4-benzpyrene into the stomachs of mice and cats has been studied with the fluorescence microscope technic. Particular attention was paid to the significance of the lipophilic and hydrophilic properties of the solvent and of the balance between these properties.

The nature of the solvent is of little importance for penetration into the mouse forestomach.

For the penetration into the glandular mucosa of the stomachs of mice and cats the nature of the solvent is decisive. Substances with only lipophilic properties are unable to effect a penetration of BP. With substances possessing both lipophilic and hydrophilic properties a weak penetration is observed if the lipophilic properties predominate, and a strong penetration if the hydrophilic properties are predominant. The solvents of the last group are miscible with water. They include the polyethylene glycols and the association colloids (soaps and soaplike substances). Also, aqueous solutions of the association colloids are able to effect a penetration of BP. It is interesting to note that the bile salt solutions also possess properties which place them in the last group of solvents.

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REFERENCES

1. EKWALL, P. Associationskolloiderna och Deras Förmåga att Bringa Främmande Ämnen i Lösning. VI. Scandinav. Chem. Congress, Lund, pp. 179–215, 1947.
2. ———. Micelle Formation in Sodium Cholate Solutions. Acta Acad. Aboensis. Math. Phys. 17, **8**:1–10, 1951.
3. EKWALL, P.; LINDSTRÖM, E. V.; and SETÄLÄ, K. The Stability of the Micelles in Bile Acid Salt Solutions of Different Acidities. Acta chem. Scandinav. (in press).
4. EKWALL, P., and SETÄLÄ, K. On the Solubilization of Carcinogenic Hydrocarbons by Association Colloids. Acta chem. Scandinav., **2**:733–39, 1948.
5. ———. Solutions of Carcinogenic Hydrocarbons in Solvents of Both Lipophilic and Hydrophilic Character. Acta de l'union internationale contre le cancer, **7**:120–25, 1950.
6. EKWALL, P.; SETÄLÄ, K.; and SJÖBLOM, L. Further Studies on the Solubilization of Carcinogenic Hydrocarbons by Association Colloids. Acta chem. Scandinav., **5**:175–89, 1951.
7. ERMALA, P.; SETÄLÄ, K.; and EKWALL, P. Gastric Absorption of 3,4-Benzpyrene. I. The Effect of Physiological Processes on Absorption. Cancer Research, **11**:753–57, 1951.

The Effect of Tumor Growth on Liver Catalase Concentration

OLETA A. KLATT AND ALFRED TAYLOR

(*Biochemical Institute of the University of Texas, and the Clayton Foundation for Research, Austin, Tex.*)

Brahn (3) observed very low liver catalase activity in human beings who had died as the result of malignant growths. Later work showed that of all the individual liver enzymes studied in cancerous rats and mice the activity of catalase was by far the most affected. Greenstein and co-workers (6, 7) have reported that with the progressive growth of the tumor there is a decrease in liver catalase activity—the activity may be as low as 1/10 to 1/20 of the original value; that the effect of the tumor growth is reversible, since on removal of the tumor the liver catalase activity returns to the original level; and that this effect is produced in general by all fast-growing tumors. Greenstein (6) and Appleman and co-workers (2) have observed that large individual variations in liver catalase occur in animals at apparently the same stage of tumor development. Both have reported that kidney and erythrocyte catalase are affected but slightly, if at all, by the presence of a tumor.

The present paper reports the results obtained in a study of the effects of the growth of transplanted and spontaneous tumors on the liver catalase concentration of mice, and the effect of the growth of mouse and rat tumor tissue cultivated in the yolk sac on the liver catalase activity of the supporting chick embryo.

The yolk sac cultivation of tumor tissue in embryonated eggs was developed in this laboratory and has been used continuously for more than 8 years (11). Tumor tissue implanted in this manner into eggs grows vigorously and uniformly. Since nontumorous rat and mouse tissue cannot be transplanted serially in eggs, the yolk sac tumor represents a pure culture of tumor cells.

It was considered that the reaction of the liver catalase concentration of chick embryos supporting the growth of rat and mouse tumor tissue could furnish data of special value in helping to resolve the problem of tumor-catalase relationship.

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MATERIALS AND METHODS

Six different tumors were used in this study: a spontaneous DBA mammary carcinoma and transplants of a DBA mammary carcinoma, DBA spindle-cell sarcoma, DBA lymphosarcoma, C3H mammary carcinoma, and rat sarcoma.

The DBA mammary carcinoma originated spontaneously in stock mice in 1941 and has been maintained by serial transplantation in embryonated eggs and mice. It has been designated as DBA mammary carcinoma 1. Transplants of this tumor in mice attain a size of 5–7 gm. in about 23 days, at which time the death of the mouse occurs. This tumor has been carried continuously by yolk sac cultivation for more than 8 years. It often has an adverse effect on the supporting embryo because of a transferred toxic effect described in a previous report (10).

The spindle-cell sarcoma designated as DBA Sarcoma 131 originated by stromal malignancy (9) from transplants of egg-cultivated DBA mammary carcinoma 1. It has been carried in mice for 122 transplant generations. This tumor grows rapidly and uniformly after transplantation, attaining a size of about 8 gm. by the eighteenth day. It has a low vascularization factor and depresses the hemoglobin concentration of the host mouse much less than does DBA mammary carcinoma 1.

The lymphosarcoma designated as Lipoid III also originated by stromal malignancy from transplants into the mouse of egg-cultivated DBA mammary carcinoma 1 (9). This tumor has a high vascularization factor and affects the hemoglobin concentrations of the host much more than does DBA mammary carcinoma 1. Transplants of this tumor in mice grow rapidly and are highly malignant to the host, which rarely survives beyond the thirteenth or fourteenth day. The tumor is difficult to weigh, as it invades the surrounding tissue, penetrating into the muscles of the body wall. It

has been carried in mice for 165 transplant generations.

The C3H mammary carcinoma originated spontaneously in stock mice and has been carried by egg and mouse transplantation for 3 years. The reaction of this tumor to egg cultivation has been described (11).

The rat sarcoma originated spontaneously in the mesenteric tissues of the visceral cavity of a stock rat. It grows more vigorously in egg cultures than in its normal host. The chick embryo appears to be little affected by this tumor, even when it attains several grams in weight.

Mice of the DBA strain received implants of DBA mammary carcinoma 1, Sarcoma 131, and Lipoid III. Spontaneous tumors were provided by exbreeder DBA females. Control mice were of the same sex and age as the corresponding experimental group.

Embryonated eggs were inoculated with tumor tissue in the yolk sacs by a technic reported in detail (11). The DBA mammary carcinoma 1, the C3H mammary carcinoma, and the rat sarcoma were cultivated in this manner.

Mice were anesthetized with ether, decapitated, the tumors and livers removed, washed with saline to remove the blood, and weighed. The livers were homogenized, and a weighed amount of the homogenate was diluted with distilled water. Chick livers and tumors were treated in a similar manner.

Catalase determinations were made by the sodium perborate method of Feinstein (5). All determinations were made on the same day that the animals were killed.

Enzyme activity was expressed in milliequivalents of sodium perborate destroyed per milligram of liver.

RESULTS

The data obtained on the effect of tumor growth on the liver concentration in mice are summarized in Tables 1 and 3. The comparable results obtained with tumor-bearing embryonated eggs are shown in Table 2.

These experimental data show that when catalase activity is calculated on the basis of units per milligram of liver, or concentration, DBA mammary carcinoma 1 induces no reduction; DBA Sarcoma 131 and spontaneous tumors, slight reductions; and Lipoid III (lymphosarcoma), a reduction of 45 per cent of the control level in mice. In the embryonic chicks a reduction in concentration is noted for all tumors. When, however, catalase activity is calculated on the basis of the whole liver, or total liver catalase per mouse or chick, values were obtained which equal or exceed those of the controls. The DBA mammary carcinoma 1 and the DBA 131 in mice are associated with total liver catalase values well above those of the controls, 130 per cent and 125 per cent, respectively. The spontaneous tumors in mice are associated with total liver catalase values that are slightly higher than those of the controls. In chick embryos bearing the C3H mammary carcinoma, the liver catalase concentration was reduced to 73 per cent of the control value, while the total liver catalase was increased to 131 per cent of the controls. Exceptions to this are the mice bearing the Lipoid III and the embryonic chicks bearing the DBA mammary carcinoma 1, in which both the concentration and the total liver catalase values were reduced. The exceptions will be discussed later.

Liver size was increased in all experimental groups, varying from 114 per cent of control, in embryonic chicks bearing the rat sarcoma, to 189 per cent of control in mice with spontaneous tumors. Even the livers of the mice bearing the DBA mammary carcinoma 1 in which no reduction in liver catalase was observed either in concentration or in total liver catalase showed an increase in weight up to 135 per cent of the controls.

DISCUSSION

The low values for liver catalase of tumor-bearing animals reported by Greenstein and co-workers and by Weil-Malherbe and Schade (12) have not been found by other investigators. Appleman and co-workers and Dounce and Shanewise (4) reported less drastic reductions in their work with rats. It was suggested by Appleman that this discrepancy may be due to differences among strains of rats, the type of tumor used, the diet and experimental technic employed. In the present experiments, by using mice of the same strain fed the same diet and eggs from the same flock of chickens, two different but equally uniform hosts were employed in which to study the effects of various tumors. Since the same experimental technic was used for all experiments, the only variable with respect to each host was the tumor.

There was a marked increase in liver size of tumor-bearing animals in all experimental groups whether or not catalase concentration was affected. Appleman *et al.* and Dounce and Shanewise reported enlargement of the livers of tumor-bearing rats with reduced catalase activities, but Greenstein and Weil-Malherbe and Schade gave no figures on liver size. Kynette *et al.* (8) in their study on the effects of egg-grown tumors on the chick embryo reported enlarged livers. Histologically, these affected chick livers were characterized

by a proliferation of cells bordering the sinusoids, hepatic cells tending to separate into small aggregates, and, in severe case, areas of necrosis and depositions of erythrocytes. There is little doubt that liver damage is a phenomenon concomitant with cancer. A study on eight hepatic functions in patients with gastrointestinal cancer by Abels *et al.* (1) revealed a high incidence of insufficiencies in these functions.

Large variations in liver catalase concentrations were observed in animals at apparently the same stage of tumor development. This is in agreement with other investigators. Large individual variations were found, however, for total catalase values as well as for catalase concentration in both tumorous and nontumorous animals of the same age.

That some tumors affect host animals more severely than others is well known. Of the four mouse-grown tumors used in this study, the Lipoid

TABLE 1

THE EFFECT OF TUMOR GROWTH ON LIVER CATALASE CONCENTRATION OF DBA MICE

(5 mice each in control and experimental groups)

Tumor	Av. tumor wt. (gm.)	Av. liver wt. (gm.) Control	Exp.	Catalase activity per mg. of liver* Control	Exp.	Catalase activity per whole liver* Control	Exp.	Catalase activity per mg. liver as per cent of av. control value	Catalase activity per whole liver as per cent of av. control value
Transplanted mammary carcinoma (DBA 1)	3.8	1.30	1.62	0.122±0.014	0.127±0.015	158.5±18.9	205.5±22.8	104.1	130.0
Transplanted spindle-cell sarcoma (DBA 131)	5.6	1.18	1.62	0.125±0.014	0.115±0.010	149.0±24.9	188.2±46.2	92.0	126.0
Spontaneous mammary carcinoma	2.9	1.63	2.09	0.166±0.018	0.146±0.018	271.8±53.9	299.8±41.8	88.0	110.3
Transplanted lymphosarcoma (Lip III)		1.18	1.80	0.155±0.011	0.070±0.011	181.9± 9.5	126.1±20.9	45.2	69.3

* Milliequivalents of $NaBO_3$.

TABLE 2

THE EFFECT OF TUMOR GROWTH ON THE LIVER CATALASE CONCENTRATION OF EMBRYONATED EGGS

(5 to 10 eggs in control and experimental groups)

Tumor	Av. tumor wt. (gm.)	Age of implant (days)	Av. liver wt. (gm.) Control	Exp.	Catalase activity per mg. of liver* Control	Exp.	Catalase activity per whole liver* Control	Exp.	Catalase activity per mg. liver as per cent of av. control value	Catalase activity per whole liver as per cent of av. control value
C3H mammary carcinoma	0.83	10	0.104	0.186	0.094	0.069	9.7†	12.8†	73.4	131.0
DBA mammary carcinoma	0.9	11	0.175	0.223	0.124±0.025	0.077±0.009	21.1±3.8	17.2±3.0	62.1	81.5
Rat sarcoma	2.3	12	0.238	0.270	0.114±0.014	0.091±0.011	26.7±0.5	24.1±1.8	78.8	90.3

* Milliequivalents of $NaBO_3$.

† Whole livers were not weighed separately.

TABLE 3

THE EFFECT OF THE GROWTH OF A TRANSPLANTED LYMPHOSARCOMA ON THE LIVER CATALASE CONCENTRATION OF MICE

(5 mice each in control and experimental groups)

Age of implant (days)	Av. liver wt. (gm.) Control	Exp.	Catalase activity per mg. of liver* Control	Exp.	Catalase activity per whole liver* Control	Exp.	Catalase activity per mg. liver as per cent of av. control value	Catalase activity per whole liver as per cent of av. control value
3	1.19	1.34	0.166±0.019	0.160±0.013	196.2±10.4	215.3±18.3	96.4	109.7
9	1.17	1.40	0.165±0.019	0.165±0.017	193.3±28.6	227.8±11.6	100.0	117.8
12	1.17	1.79	0.155±0.011	0.070±0.011	181.9± 9.5	126.1±20.9	45.2	69.3
15	1.26	2.26	0.162±0.022	0.062±0.014	208.1±30.5	126.7±12.1	38.3	60.9

* Milliequivalents of $NaBO_3$.

III was the only one that was associated with a reduction of total liver catalase. This highly malignant lymphosarcoma is more invasive than are other transplanted tumors, and it stimulates unusual vascularization of the neoplastic area, whereby it robs the host of a large amount of blood. Cachexia appears on about the seventh day after tumor inoculation. From the data given in Table 3 it will be noted that total liver catalase was not reduced until the twelfth day, or shortly before the death of the animal. Decreased liver catalase could not have been a causative factor in the cachexia, since it was not found until after the animal had been severely affected.

Of the egg-grown tumors, the DBA mammary carcinoma was associated with a reduction in total liver catalase. The general toxic effects of this tumor on the chick embryo have been described by Taylor and Carmichael (10). The slightly lower total catalase values for the chicks associated with the rat sarcoma can be attributed to a reduction in size of the tumor-bearing embryo. It has been shown that increasing tumor size is associated with a decrease in the weight of the host chick (8) which would result in a corresponding reduction in the size of the liver. This is not regarded as a reduction in total liver catalase.

The liver catalase concentration may not have been directly affected by the presence of a tumor in the host. It seems more probable that the changes observed in liver catalase activity in association with tumor growth were mediated by the fact that the liver of tumor-bearing animals increases in size without a corresponding increase in liver tissue (8). Consequently, a milligram of liver from a tumor-bearing animal has less active liver tissue in it than a milligram of control liver. The result was that the liver catalase activity per milligram tended to be lower in association with tumor growth. On the other hand, the experimental livers were enough larger than the controls to compensate for this factor and to bring the total liver catalase activity up to or above that of the livers from the nontumorous animals. This may explain the results of other investigators, who found a reduction in liver catalase concentration in tumor-bearing animals but only slight or no reduction in kidney and erythrocyte catalase activity from the same animal. The kidneys enlarge only slightly in association with tumor growth.

SUMMARY

A study was made of the effect of tumor growth on the concentration of liver catalase in mice and in chick embryos supporting the growth of yolk sac tumors.

A total of 80 DBA mice was used, the experimental groups of which bore transplants of a mammary carcinoma, a sarcoma, a lymphosarcoma, and a spontaneous mammary carcinoma.

Tests were completed on the livers of 44 chick embryos. The tumor-bearing eggs had been inoculated in the yolk sac with a DBA mammary carcinoma, a C3H mammary carcinoma, and a rat sarcoma.

There was a reduction in liver catalase activity in association with tumor growth in both the embryo and mouse series of experiments when the enzyme activity was based on unit weight of liver for experimental and control group.

The total liver catalase of the tumor-bearing mice and of the embryos supporting tumors tended to be unaffected or slightly higher than that of the corresponding controls.

It is suggested that, since the presence of a tumor in an animal is associated with changes in the liver which result in an increase in weight without a corresponding increase in active liver tissue, the effect is to dilute the per milligram catalase concentration. The increase in liver size compensates for the dilution effect, so that the total liver catalase activity is relatively unaffected.

REFERENCES

1. ABELS, J. C.; REKERS, P. E.; PACK, G. T.; and RHOADS, C. P. Metabolic Studies in Patients with Cancer of the Gastrointestinal Tract. II. Hepatic Disfunctions. Ann. Internal. Med., **16**:221–40, 1942.
2. APPLEMAN, D.; SKAVINSKI, E. R.; and STEIN, A. M. Catalase Studies on Normal and Cancerous Rats. Cancer Research, **10**:498–505, 1950.
3. BRAHN, B. Further Researches on the Enzymes in the Liver of the Cancerous. Sitzber. kgl. preuss. Akad. Wiss. **20**:478–81, 1916.
4. DOUNCE, A. L., and SHANEWISE, R. P. Liver Catalase of Tumor-bearing and Leprous Rats. Cancer Research, **10**: 103–7, 1950.
5. FEINSTEIN, R. N. Perborate as Substrate in a New Assay of Catalase. J. Biol. Chem., **180**:1197–1202, 1949.
6. GREENSTEIN, J. P. Titration of the Liver Catalase Activity of Normal and Tumor-bearing Rats and Mice. J. Nat. Cancer Inst., **2**:525–30, 1942.
7. GREENSTEIN, J. P.; JENRETTE, W. V.; and WHITE, J. The Liver Catalase Activity of Tumor-bearing Rats and the Effect of Extirpation of the Tumors. J. Nat. Cancer Inst., **2**:283–99, 1941.
8. KYNETTE, A.; TAYLOR, A.; and THOMPSON, R. C. Effects of Egg-grown Heterologous Tumor Tissue on the Chick Embryo. University of Texas Publication, No. 4507, pp. 65–73, 1945.
9. TAYLOR, A., and CARMICHAEL, N. Stromal Malignancy in Mouse-grown Transplants of Egg-cultivated Mouse Mammary Carcinoma. Cancer Research, **7**:78–87, 1947.
10. ———. The Effect on the Embryo of Continued Serial Tumor Transplantation in the Yolk Sac. *Ibid.*, **9**:498–503, 1949.
11. TAYLOR, A.; CARMICHAEL, N.; and NORRIS, T. A Further Report on Yolk Sac Cultivation of Tumor Tissue. Cancer Research, **8**:264–69, 1948.
12. WEIL-MALHERBE, H., and SCHADE, R. Studies on the Liver Catalase of Normal and Cancerous Rats. Biochem. J., **43**:118–25, 1948.

The Effect of Tumor Implants on Chick Embryo Liver Catalase Activity*

EDWIN R. SKAVINSKI AND ABRAHAM M. STEIN

(College of Agriculture, University of California, Los Angeles, Calif.)

Several possible explanations have been suggested for the drastic decreases in liver catalase activity produced by tumor implants in mice and rats (1, 4, 5, 9). Since no specific mechanism has been demonstrated, the alternative hypotheses and evidence are presented below:

1. There may be an actual interference with the synthesis of catalase necessary to maintain the normal concentration in the liver. However, in a previous paper (1) it was shown that a significant number of rats failed to show a decreased catalase activity in the presence of large tumors.

2. One possible mechanism of interference is the production by the tumor of some toxic substance which affects the liver catalase activity. Recent work (6) has led to the extraction of a fraction of low molecular weight from tumors which, when injected intraperitoneally, lowered liver catalase activity of normal mice.

3. There may be abstraction from the circulation of some substance necessary for the maintenance of a normal liver catalase activity.

4. Because of the increased protein requirement of the animal with a growing tumor, experiments have been performed by varying the level of dietary protein (9). Increased levels of protein had little effect on the liver catalase of tumor-bearing rats.

This work was undertaken to determine the effect of tumor implants upon the liver catalase activity during embryonic development. It was felt that the avian egg would provide a fully adequate system for testing the effect of the tumor on liver catalase, since it is free from the variable of a possible, but undetected, essential limiting substance in standard laboratory diets. Furthermore, the physiological adaptability of the embryo might allow it to adjust its metabolic processes to meet the demands imposed by both the normal developmental process and the growth of the tumor.

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MATERIALS AND METHODS

Embryonated New Hampshire Red eggs, supporting growth of implants of Brown-Pearce carcinoma and Jensen fibrosarcoma on the chorioallantoic membrane, were used for the liver catalase measurements. The desired tumor tissue was implanted into eggs which had been incubated for 8 days at 37.5° C. The procedure was that reported by Schechtman *et al.* (8).[1] The embryos were obtained after the eighteenth day of incubation. Sham-operated groups and embryos inoculated with tumor tissue, but showing no growth of the implant, were used as control groups to determine the normal enzymatic activity.

The tumor was dissected from the chorioallantoic membrane and weighed; the liver was removed, weighed, sealed, and immediately frozen. The weight of the remaining carcass was also determined. In most cases the liver samples were pooled into groups classified according to the amount of tumors found. The pooled or single liver samples were homogenized at 0° C. in M/15 phosphate buffer at pH 7.0. Catalase activity was determined by the procedure reported in a previous publication (1); aliquots of the homogenate were analyzed for nitrogen by the customary semi-micro-Kjeldahl method.

RESULTS

Sixty-two chick embryos were collected and separated into groups as shown in Table 1. Nitrogen and catalase activity values are expressed on various bases to facilitate a more accurate interpretation of the data. A portion of the data is further presented in Chart 1. The changes produced by the growth of the tumor may be summarized as follows:

1. An increase in rate of growth of the liver is effected. This is accompanied by an approximately proportionate increase in the total liver nitrogen.

[1] We are indebted to Dr. A. M. Schechtman, Ellis C. Berkowitz, and Melvin J. Cohen for their generosity and aid in providing the experimental material used for the analytical determinations.

TABLE 1

THE EFFECTS OF TUMOR IMPLANTS ON THE CHICK EMBRYO LIVER

	Sham-operated control animals		Nontake group	Brown-Pearce carcinoma 0–0.5 per cent	Brown-Pearce carcinoma 0.8–5 per cent			Jensen sarcoma 0.8–3.5 per cent				Jensen sarcoma 4.7–7.3 per cent					Jensen sarcoma 9.4–13.9 per cent		
	(4)*	(4)	(3)	(8)	(14)	(11)	(3)	(1)	(2)	(1)	(2)	(2)	(1)	(1)	(1)	(1)	(1)	(1)	(1)
Liver, weight per cent	2.68	2.62	2.60	3.37	3.21	3.49	3.54	2.67	3.28	3.41	3.70	3.50	4.36	3.92	3.54	3.51	4.57	4.02	3.27
Average value of groups	2.65					3.41			3.27					3.77				3.95	
Liver nitrogen, mg/100 gm embryo weight	56	62	59	77	81	87	89	67	88	88	90	85	115	101	79	77	112	99	83
Average	59					86			83					91				98	
Liver nitrogen, mg/gm liver	21	24	23	23	25	25	25	25	27	26	24	24	26	26	22	22	25	25	25
Average	22.5					25			26					24				25	
Liver catalase activity units/ 100 gm embryo wt.	90	95	89	81	105	115	106	85	161	137	116	119	142	107	104	86	132	121	110
Average	92.5					109			125					112				121	
Liver catalase activity units/ mg Liver N	1.59	1.53	1.49	1.06	1.30	1.33	1.20	1.47	1.85	1.56	1.30	1.40	1.24	1.04	1.32	1.14	1.18	1.22	1.33
Average	1.56					1.28			1.55					1.23				1.24	
Liver catalase activity units/ gm liver	33	35	34	26	33	33	30	37	49	40	31	34	33	27	29	25	29	30	34
Average	34					32			39					30				31	
Average tumor weight per cent group			0	0.2	1.6	1.8	4	1.8	1.0	2.9	3.2	4.7	6.8	7.1	7.3	5.6	10.8	13.9	9.4
Range of group tumor size in percentages			0	0.01–0.5	0.8–2.4	0.7–3.7	3.5		0.8–1.4		3–3.5								

* The number of animals in the groups is indicated by parentheses.

2. There is a greater proportionate increase in liver nitrogen than in liver catalase activity.

3. Liver catalase activity increases if calculated per hundred grams of body weight but shows values below those of the control groups when calculated on a unit nitrogen basis or on a fresh tissue weight basis.

4. The stimulation of liver growth and nitrogen incorporation occurs with small amounts of tumor growth. Stimulation of nitrogen incorporation in the liver is not directly proportional to tumor mass.

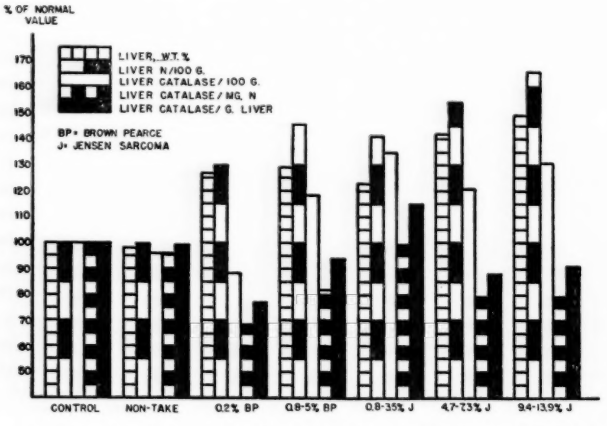

CHART 1.—Tumor implants and their effects on the chick embryo liver.

DISCUSSION

The data illustrate the importance of the selection of a suitable basis for the interpretation of changes in enzyme activity. Thus, the expression of enzyme activity per unit quantity of liver nitrogen would indicate that the presence of the tumor decreased the liver catalase activity. On the other hand, when expressed per unit weight of embryo, the data indicate an actual increase in total liver catalase activity in the embryo supporting growth of a tumor. This discrepancy is readily explained by the significantly larger liver and slightly elevated concentration of nitrogen present in the liver of the tumor-bearing chick embryo.

We feel that the latter mode of expression is more valid, since it reflects the total quantity of liver enzyme available to the organism; we therefore interpret the data obtained in this system as not supporting the hypothesis of an interference with liver catalase synthesis by tumor growth. Although increased liver nitrogen suggests an increased number of liver cells, one cannot draw conclusions as to the quantities of catalase per cell without a counting method. It is of interest that initially increased levels of liver catalase activity were reported by Greenstein *et al.* (5) in rats bearing Hepatoma 31.

The increases in liver fresh weight and nitrogen are interpreted as an increase in the rate of growth of the liver caused by the influence of the tumor. This is provisionally interpreted as a readjustment on the part of the liver in an effort to meet the metabolic requirements of the tumor.

The data presented herein indicate increased total amounts of liver catalase in the chick embryo which is supporting the growth of a tumor. It is suggested that the effect of the tumor is to create either qualitative or quantitative changes in intermediary metabolism patterns. The adult rat may not mediate these metabolic changes as readily as the chick embryo. Evidently the embryo possesses the nutritional adequacy and/or the synthetic ability to mediate a more rapid, or a different pattern of, metabolism in response to the tumor growth.

Friedberg *et al.* (3) and Borsook *et al.* (2) have shown that the rate of amino acid incorporation into proteins is much greater in the fetus than in the adult animal. Zamecnik *et al.* (10) have also shown that the rate of incorporation of labeled amino acids into the proteins of surviving hepatoma nodules was 7 times that of slices of normal livers and $2\frac{1}{2}$ times that of slices from the nonmalignant portions of the hepatoma-containing livers.

The data do not support a tendency to establish an enzyme level in the liver of the host similar to the low catalase level found in the growing tumor (5). It is felt that it would be reasonable to assume that the tumor causes an increase in the quantitative demand for certain enzymes beyond the normal requirements of the animal. Rosenthal *et al.* (7) have shown that the rate of restoration of liver arginase activity after partial hepatectomy of rats was geared to the endogenous protein catabolism of the animal and paralleled urinary nitrogen excretion. Arginase activity restoration was greater in the case of protein-starved animals. Thus, one could explain increased, decreased, and normal enzyme activity levels in terms of the metabolic demands imposed by tumor growth and capacity of the animal to synthesize enzymes. This, of course, assumes the synthesis and destruction of enzyme molecules in the processes of intermediary metabolism. Insufficient data prevent the evaluation of results in terms of new qualitative types of metabolism patterns.

Future studies of the enzyme patterns in the tumor-bearing chick embryo may yield a more accurate interpretation of the effects of tumors on enzyme systems.

SUMMARY

1. The effects of chorioallantoic implants of Brown-Pearce carcinoma and Jensen fibrosarcoma

on liver catalase activity in the chick embryo have been studied.

2. The expression of liver catalase activity on a unit nitrogen basis shows decreased enzymatic activity, but the total quantity of liver catalase per unit weight of chick embryo plus tumor shows increased activity. The data suggest that tumor growth does not interfere with catalase synthesis.

3. Tumor growth stimulates incorporation of liver nitrogen to a greater extent than the increase in total liver catalase activity.

REFERENCES

1. APPLEMAN, D.; SKAVINSKI, E. R.; and STEIN, A. M. Catalase Studies on Normal and Cancerous Rats. Cancer Research, **10**:498–505, 1950.
2. BORSOOK, H.; DEASY, C. L.; HAAGEN-SMIT, A. J.; KEIGHLEY, G.; and LOWY, P. H. Uptake of Labeled Amino Acids by Tissue Proteins *in Vitro*. Fed. Proc., **8**:589–602, 1949.
3. FRIEDBERG, F.; SCHULMAN, M. P.; and GREENBERG, D. M. The Effect of Growth on the Incorporation of Glycine Labeled with Radioactive Carbon into the Protein of Liver Homogenates. J. Biol. Chem., **173**:437–38, 1948.
4. GREENSTEIN, J. P. Titration of the Liver Catalase Activity of Normal and of Tumor-bearing Rats and Mice. J. Nat. Cancer Inst., **2**:525–30, 1942.
5. GREENSTEIN, J. P.; JENRETTE, W. V.; and WHITE, J. The Liver Catalase Activity of Tumor-bearing Rats and the Effect of Extirpation of the Tumors. J. Nat. Cancer Inst., **2**:283–91, 1941.
6. NAKAHARA, W., and FUKUOKA, F. Toxohormone: A Characteristic Toxic Substance Produced by Cancer Tissue. Gann, **40**:45–71, 1949.
7. ROSENTHAL, O.; ROGERS, C. S.; VARS, H. M.; and FERGUSON, C. C. Arginase, Adenosine Pyrophosphatase, and Rhodanese in Regenerating Rat Liver. J. Biol. Chem., **189**:831–43, 1951.
8. SCHECHTMAN, A. M.; COHEN, M. J.; and BERKOWITZ, E. C. Culture of Brown-Pearce Carcinoma in the Embryonated Egg. Proc. Soc. Exper. Biol. & Med., **74**:784–89, 1950.
9. WEIL-MALHERBE, H., and SCHADE, R. Studies on the Liver Catalase of Normal and Cancerous Rats. Biochem. J., **43**: 118–25, 1948.
10. ZAMECNIK, P. C.; FRANTZ, I. D.; LOFTFIELD, R. B.; and STEPHENSON, M. L. Incorporation *in Vitro* of Radioactive Carbon from Carboxyl-labeled DL-Alanine and Glycine into Proteins of Normal and Malignant Rat Livers. J. Biol. Chem., **175**:299–314, 1948.

Phosphorus Metabolism in Resting and Pregnancy-stimulated Mammary Glands and in Spontaneous Mammary Carcinomas of Mice*†

S. Albert, R. M. Johnson, and Marjorie S. Cohan

(*Richard Cohn Radiobiology Laboratory, Detroit Institute of Cancer Research, Detroit 1, Mich.*)

Tumors have been found to contain about the same amounts of phosphorus characteristic of normal tissue (4, 5, 16), but its turnover in tumors is often significantly greater than normal (1, 5, 18) and in liver tumors tends to resemble that of the homologous tissue undergoing restoration following partial hepatectomy (1). In general, however, tumors have not been compared to the homologous tissues under the influence of a physiological stimulation. In the present study the amounts of phosphorus in the acid-soluble, phospholipid, phosphoprotein, DNA[1] and PNA[1] fractions, and the uptake of radioactive phosphorus by these fractions of spontaneous mammary gland carcinomas of the mouse were compared to those in the corresponding fractions of resting and pregnancy-stimulated mammary glands.

MATERIALS AND METHODS

The mice used were females of the DBA and C3H strains,[2] 20–42 gm. in weight, and 4–20 months of age. They were fed Purina Mouse Chow and water *ad libitum*. Resting mammary glands were removed from the mice at least 10 days after delivery of their second litters, which had not been permitted to suckle. Stimulated glands were removed from pregnant females 3–5 days prior to the expected termination of their third pregnancy. Nineteen primary spontaneous mammary gland carcinomas were obtained from adult nonpregnant mice and freed of excess blood and necrotic material before use.

The radioactive phosphorus,[3] as $H_3P^{32}O_4$, was neutralized with sodium hydroxide. An aliquot was removed and diluted to the desired volume with distilled water. From this solution standards were prepared by evaporating 0.5-ml. aliquots of the diluted solution on soft glass planchets 24 mm. in diameter. The injected dose was calculated from these standards. Approximately 26 (range 20–33)[4] μc. of phosphorus was administered as a single subcutaneous dose to each mouse.

The animals were killed by cervical dislocation at 1, 4, 17, or 48 hours after injection of radioactive phosphorus, and the tumors and mammary glands were rapidly dissected out and weighed. Portions of the appropriate tissues were fixed in 10 per cent formalin, sectioned, and stained with hematoxylin and eosin for microscopic examination. The tissues for chemical analysis were immediately placed in 10 per cent TCA[1] at 1°–2° C., and homogenized in an all-glass homogenizer of the Potter-Elvehjem type (11). Aliquots of these homogenates were taken for total nitrogen and phosphorus analyses. The remainder, equivalent to 0.4–0.8 gm. of tissue, was fractionated essentially according to the method of Schmidt and Thannhauser (13, 15).

The following change in this method, which permitted a more complete separation of phosphoprotein and PNA phosphorus, was developed. To 7–10 ml. of the DNA–protein-free supernatant so-

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[1] For the sake of convenience, the abbreviations for desoxypentosenucleic acid and pentosenucleic acid used herein will be DNA and PNA, respectively; DNA protein = desoxynucleoprotein; TCA = trichloroacetic acid.

[2] These strains originated at the Roscoe B. Jackson Memorial Laboratory and have been maintained subsequently by brother-sister mating at the New York State Institute for the Study of Malignant Disease and the Detroit Institute of Cancer Research by Dr. William S. Murray and others.

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[3] The radioactive phosphorus used in these experiments was furnished by the Oak Ridge National Laboratory, Oak Ridge, Tenn.

[4] These values have been corrected, since our counting arrangement is approximately 21 per cent efficient.

lution in a 15-ml. centrifuge tube was added 0.2 ml. of 15 per cent $MgCl_2$, containing an equimolar quantity of NH_4Cl, and 1 drop of 1 per cent thymolphthalein in ethanol. The mixture was chilled, placed in an ice-water bath, and concentrated NH_4OH was added dropwise with vigorous stirring until a blue end-point was reached. The mixture was stirred for an additional 10 minutes with a mechanically driven glass rod and was stored in a refrigerator (3°–4° C.) for at least 4 hours. It was then centrifuged at 0°–3° C. The supernatant solution was removed, and the precipitate (to be referred to as the first phosphoprotein precipitate) washed 3 times with ice-cold 10 per cent NH_4OH. The supernatant solution and washings were combined, and acidified with a slight excess of 6 N HCl. After adding 160 μg. of carrier phosphorus, as Na_2HPO_4, orthophosphate was reprecipitated as described above (to be referred to as the second phosphoprotein precipitate).

The addition of carrier phosphate invalidated direct chemical determinations of both the remaining phosphoprotein and the PNA phosphorus. To determine the amount of total phosphoprotein phosphorus, the phosphorus in the first phosphoprotein precipitate was determined chemically. The specific activity of the phosphoprotein phosphorus was then calculated from the total phosphorus and radioactivity values of the first phosphoprotein precipitate. The amount of phosphoprotein phosphorus present in the second phosphoprotein precipitate was determined by dividing the amount of radioactivity found in this fraction by the calculated specific activity of the first precipitate. The phosphorus thus determined plus that found in the first phosphoprotein precipitate represented total phosphoprotein phosphorus.

PNA phosphorus was calculated by subtracting the phosphoprotein phosphorus from the total phosphorus of the supernatant. The radioactivity content of this fraction was determined as the difference between the activities of the DNA–protein-free supernatant and the phosphoprotein fraction. The calculated values differed from the values determined by radioactivity assay by only 6 per cent.

The nitrogen content of the homogenate, the acid-soluble and the lipid fractions were determined by the usual micro-Kjeldahl procedure (9). The value for protein nitrogen was obtained by subtracting the value of the acid-soluble and lipid fractions from the amount found in the homogenate. Phosphorus was determined according to the method of Fiske and SubbaRow (6), after a 70 per cent perchloric acid digestion of the tissues.

Radioactivity was determined with an end-window counter and a standard scaling assembly.[5] The expression "concentration coefficient," slightly modified from that suggested by Schulman and his co-workers (17, 18), has been used to indicate the uptake of radioactivity and was calculated in the following manner:

$$\frac{\text{counts/min found in fraction}/\mu\text{g phosphorus in fraction}}{\text{counts/min injected}/\mu\text{g of body weight}}$$

In this expression the counts per minute injected were recalculated from standards counted on the same day as the experimental samples in order to avoid correcting for decay of the radioactivity in them.

RESULTS

The technics employed in these experiments resulted in an over-all recovery of phosphorus in the various fractions equivalent to 100 ± 1.1 per cent of the total found in the homogenates. The total radioactivity recovered was 112 ± 1.8 per cent.

The amounts of phosphorus found in the various fractions calculated on a protein nitrogen basis are shown in Table 1. Stimulated mammary glands and mammary carcinomas contained approximately the same amount of phosphorus, 137 and 144 μg/mg of protein nitrogen, respectively, as compared with only 92 μg/mg in resting glands. The higher phosphorus content of the carcinomas and pregnancy-stimulated glands was not accounted for by proportional increments in all fractions. The greatest increases occurred in the nucleic acid fractions. Approximately the same amounts of PNA phosphorus were found in the carcinomas and pregnancy-stimulated glands; in both tissues, the values were more than 3 times those for the resting glands. Three times as much DNA phosphorus was found in the carcinomas as in the resting glands, and more than twice as much as in the stimulated ones. There was a higher concentration of acid-soluble, phospholipid,and phosphoprotein phosphorus in stimulated glands than in either resting glands or carcinomas. The increase was only slight, however, The concentration of phosphoprotein phosphorus was of the same order in resting glands and carcinomas, and that of the phospholipid was lowest in the carcinomas. In the acid-soluble fraction of the tumors the phosphorus concentration was intermediate between that of the two types of mammary issue.

The concentration coefficients in all mammary tissues and carcinomas studied were highest in the

[5] A Tracerlab TGC-1 Geiger counter and a Radioactive Products Co. Raychronometer were used for assaying radioactivity. Those Geiger counts that did not exceed the background by 10 were not considered significant, 10 counts being approximately 3 times the standard error of the background.

acid-soluble fraction and next highest in the phosphoprotein. The lowest values were in the DNA fraction, and those of the phospholipid and PNA fractions were intermediate (Table 2).

The concentration coefficients in all fractions of the pregnancy-stimulated mammary glands were, in general, greater than those of the resting glands or carcinomas. The concentration coefficients of the various fractions of tumors were not consistently higher or lower than those of similar fractions of the resting mammary glands. However, they tended to be higher in the resting glands soon after injection and in the carcinomas later.

The changes in the concentration coefficients for any given fraction over the experimental time period were similar for all three tissues. In the acid-soluble and phosphoprotein fractions they increased relatively rapidly, apparently reached maxima prior to 4 hours, and then fell gradually. In the phospholipid fractions the concentration coefficients increased rather slowly, reached maximum values after about 17 hours, and either maintained this level or fell slightly thereafter. In the nucleic acid fractions the coefficients increased very slowly throughout the entire experimental period (Table 2).

The spontaneous mammary carcinomas exhibited varying degrees of differentiation, as indicated by the tendency of the neoplastic cells to form glandular or pseudo-glandular structures. However, no general correlation was found between the level of differentiation and the amount of phosphorus in the several fractions of the tumor or their uptake of radioactive phosphorus.

DISCUSSION

The fact that the carcinomas of the mammary gland investigated in these experiments contained more phosphorus in the acid-soluble, DNA, and PNA fractions, and less in the phospholipid, than did the resting glands corresponds in general to the reports for brain and lung tumors and hepatomas (4, 5, 8, 14, 16, 19) but differs in certain respects from results obtained with gastric and skin carcinoma and hepatoma (2, 7, 16, 18).

The relatively high concentrations of DNA

TABLE 1

PHOSPHORUS DISTRIBUTION IN MOUSE MAMMARY GLANDS AND MAMMARY GLAND CARCINOMAS*

Tissue	Acid-soluble	Phospho-lipid	Phospho-protein	DNA	PNA	Total phosphorus
Resting mammary gland	33.8±1.1†	30.0±0.91	3.5±0.12	12.8±0.33	11.4±0.70	91.9
Pregnancy-stimulated mammary gland	44.9±1.1	33.3±0.81	5.2±0.40	18.8±0.33	34.7±1.0	136.9
Mammary gland carcinoma	39.8±0.97	25.8±0.74	3.1±0.11	38.0±1.4	37.7±0.10	143.9

* Micrograms of phosphorus per milligram of protein nitrogen.

† Standard errors of the mean values.

TABLE 2

THE CONCENTRATION COEFFICIENTS OF VARIOUS PHOSPHORUS-CONTAINING FRACTIONS OF RESTING AND PREGNANCY-STIMULATED MAMMARY GLANDS AND MAMMARY GLAND CARCINOMAS*

Fraction	Tissues	1 Hour	4 Hours	17 Hours	48 Hours
Acid-soluble	Resting glands	1,055 (462)	948 (107)	466 (45)	294 (80)
	Stimulated glands	1,113 (214)	1,228 (45)	639 (159)	449 (73)
	Carcinomas	733 (96)	579 (107)	495 (70)	286 (29)
Phospholipid	Resting glands	43 (13)	145 (49)	188 (16)	213 (43)
	Stimulated glands	62 (13)	265 (63)	436 (134)	404 (54)
	Carcinomas	22 (3)	68 (27)	374 (69)	379 (39)
Phosphoprotein	Resting glands	326 (188)	558 (143)	205 (19)	116 (29)
	Stimulated glands	579 (104)	927 (246)	586 (233)	385 (37)
	Carcinomas	393 (62)	509 (167)	512 (147)	195 (17)
DNA	Resting glands	1†	11 (6)	4 (2)	8‡
	Stimulated glands	6‡	73 (26)	57 (16)	214 (46)
	Carcinomas	4 (3)	11 (4)	51 (17)	113 (38)
PNA	Resting glands	100 (48)	151 (14)	150 (15)	228 (104)
	Stimulated glands	45 (7)	206 (65)	261 (74)	391 (62)
	Carcinomas	35 (10)	83 (14)	220 (41)	269 (46)

* Each value is the average of three to seven observations. The average deviation is indicated in parentheses.

† One out of four animals was found to have a significant amount of radioactivity in this fraction.

‡ Two out of four animals in the case of the resting glands and two out of three in the stimulated ones had significant amounts of radioactivity in this fraction.

phosphorus found in the mammary tumors suggest either that the amount of DNA per carcinoma cell was increased or that there was an increased cellularity. The recent work of Cunningham, Griffin, and Luck (3) indicated that increased concentrations of DNA in livers showing preneoplastic or neoplastic changes are due to increased cellularity and also to polyploidy. Assuming increased cellularity as the major factor accounting for the increase in DNA in the present experiments, the similarity in the values of the acid-soluble, lipid, and phosphoprotein phosphorus in the tumors and in resting glands suggests that the average neoplastic cell either had a smaller amount of cytoplasm or possessed lower cytoplasmic concentrations of these substances than did the average normal resting cell. Microscopically, the tumor cells appeared to have a smaller cytoplasmic volume than resting cells, supporting the first of these two possibilities.

If it is assumed that mammary tissue is similar to liver tissue in that there is no change in the DNA content of the nucleus when the cell becomes cancerous (3, 10, 12), we may reduce the data of Table 1 to a cell basis by comparing them to DNA (Table 3).

Since the amounts of acid-soluble, phospholipid, and phosphoprotein phosphorus and protein nitrogen in tumors are about one-third the values found in resting glands, one might infer that the cytoplasmic volume of the cells of the former were approximately one-third of that of the latter. Applying the same reasoning to the case of the stimulated glands, it would appear that the cytoplasmic volume of their cells is about two-thirds that of the resting mammary gland cells. If these relationships are true, it follows from these data that there are no outstanding differences in the amounts of either protein nitrogen or acid-soluble and phospholipid phosphorus per cell among these three tissues. Furthermore, while the amount of protein phosphorus is the same in resting gland and tumor cells, it is higher in the cells of the stimulated glands. Last, there is 3 times as much PNA per tumor cell as there is in a resting mammary gland cell, and there is a similar increase in the stimulated gland cells.

The rate of uptake of radioactive phosphorus in the acid-soluble fraction was found to be lower in the carcinomas than in either resting or stimulated glands. In the phosphoprotein fraction of resting glands and carcinomas the rates were nearly equal, both being lower than in the stimulated glands. In the phospholipid, DNA, and PNA fractions the rate of uptake was lowest in the resting glands, highest in the stimulated ones, and intermediate in the carcinomas. These results suggest that the development of cancer in the mammary glands of C3H and DBA mice either produced no change in the rate of uptake of radioactivity in the phospholipid, phosphoprotein, DNA, and PNA fractions or increased it only within the limits found in the mammary glands physiologically stimulated by pregnancy.

TABLE 3

PHOSPHORUS AND NITROGEN OF MOUSE MAMMARY GLANDS AND MAMMARY GLAND CARCINOMAS*

Tissue	Acid-soluble phosphorus	Phospholipid phosphorus	Phosphoprotein phosphorus	PNA phosphorus	Protein nitrogen
Resting mammary gland	2.74	2.32	0.27	0.89	78.2
Pregnancy-stimulated mammary gland	2.38	1.77	0.27	1.85	53.2
Mammary gland carcinoma	1.05	0.68	0.08	0.99	26.3

* Figures shown are micrograms of fraction phosphorus and tissue protein nitrogen per microgram of DNA phosphorus.

SUMMARY

1. Radioactive phosphorus, as $Na_2HP^{32}O_4$, was administered to normal and pregnant female mice and to females bearing mammary gland carcinomas, and the animals were sacrificed at 1, 4, 17, or 48 hours after injection. The mice were of the cancer-susceptible C3H and DBA strains. The amounts of phosphorus and radioactivity were determined in the acid-soluble, phospholipid, phosphoprotein, DNA, and PNA fractions.

2. The concentration of phosphorus in the DNA fraction of the carcinomas was increased threefold over that found in the resting glands, and twofold over that found in the stimulated glands. On the basis of the findings of other workers, it was concluded that this increased DNA phosphorus concentration denotes increased cellularity rather than increased nuclear concentration.

3. The concentration of phosphorus in the acid-soluble and PNA fractions of the mammary gland carcinomas more closely resembled that found in the pregnancy-stimulated glands, while that in the phospholipid and phosphoprotein fractions resembled that found in the resting glands.

4. The uptake of radioactivity in all fractions of the carcinomas, except the acid-soluble, was higher than in resting glands. In all fractions it was lower than in the physiologically stimulated glands.

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REFERENCES

1. Brues, A. M.; Tracy, M. M.; and Cohn, W. E. Nucleic Acids of Rat Liver and Hepatoma: Their Metabolic Turnover in Relation to Growth. J. Biol. Chem., **155**:619–33, 1944.
2. Costello, C. J.; Carruthers, C.; Kamen, M. D.; and Simoes, R. L. The Uptake of Radiophosphorus in the Phospholipid Fraction of Mouse Epidermis in Methylcholanthrene Carcinogenesis. Cancer Research, **7**:642–46, 1947.
3. Cunningham, L.; Griffin, A. C.; and Luck, J. M. Polyploidy and Cancer. The Desoxypentosenucleic Acid Content of Nuclei of Normal, Precancerous, and Neoplastic Rat Tissues. J. Gen. Physiol., **34**:59–63, 1950.
4. Davidson, J. N., and Waymouth, C. Tissue Nucleic Acids. 3. The Nucleic Acid and Nucleotide Content of Liver Tissue. Biochem. J., **38**:379–85, 1944.
5. Erickson, T. C.; Larson, F.; and Gordon, E. S. The Uptake of Radioactive Phosphorus by Malignant Brain Tumors. J. Lab. & Clin. Med., **34**:578–91, 1949.
6. Fiske, C. H., and SubbaRow, Y. The Colorimetric Determination of Phosphorus. J. Biol. Chem., **66**:375–400, 1925.
7. Fujiwara, J.; Nakahara, W.; and Kishi, S. Comparison of Chemical Composition between Hepatoma and Normal Liver Tissues. III. Phosphorus Compounds, Cholesterol, and Fatty Acids. Gann, **31**:51–63, 1937.
8. Lustig, B. Die chemische Zusammensetzung normaler und pathologisch veränderter Organe. I. Mitteilung: die Zusammensetzung der normalen, carcinomatösen und sarkomatösen Lunge. Biochem. Ztschr., **284**:367–75, 1936.
9. Ma, T. S., and Zuazaga, G. Micro-Kjeldahl Determination of Nitrogen. A New Indicator and an Improved Rapid Method. Ind. Eng. Chem., Anal. Ed. **14**:280–82, 1942.
10. Mark, D. D., and Ris, H. A Comparison of Desoxyribonucleic Acid Content in Certain Nuclei of Normal Liver and Liver Tumors. Proc. Soc. Exper. Biol. & Med., **71**: 727–29, 1949.
11. Potter, V. R., and Elvehjem, C. A. A Modified Method for the Study of Tissue Oxidations. J. Biol. Chem., **114**: 495–504, 1936.
12. Price, J. M.; Miller, E. C.; Miller, J. A.; and Weber, G. M. Studies on the Intracellular Composition of Livers from Rats Fed Various Aminoazo Dyes. II. 3-'Methyl-, 2'-Methyl-, and 2-Methyl-4-dimethylaminoazobenzene and 4'-Fluoro-4-dimethylaminoazobenzene. Cancer Research, **10**:18–27, 1950.
13. Schmidt, G., and Thannhäuser, S. J. A Method for the Determination of Desoxyribonucleic Acid, Ribonucleic Acid, and Phosphoprotein in Animal Tissues. J. Biol. Chem., **161**:83–89, 1945.
14. Schneider, W. C. Phosphorus Compounds in Animal Tissues. II. The Nucleic Acid Contents of Homologous Normal and Cancer Tissues. Cancer Research, **5**:717–21, 1945.
15. ———. Phosphorus Compounds in Animal Tissues. III. A Comparison of Methods for the Estimation of Nucleic Acid. J. Biol. Chem., **164**:747–51, 1946.
16. Schneider, W. C., and Klug, H. L. Phosphorus Compounds in Animal Tissues. IV. The Distribution of Nucleic Acids and Other Phosphorus-containing Compounds in Normal and Malignant Tissues. Cancer Research, **6**:691–94, 1946.
17. Schulman, J., Jr., and Falkenheim, M. Review of Conventions in Radiotracer Studies. An Analysis of Expressions Found in the Literature with Suggestions for Conventions for Future Use. Nucleonics, **3**:13–23, 1948.
18. Schulman, J., Jr.; Falkenheim, M.; and Gray, S. J. The Phosphorus Turnover of Carcinoma of the Human Stomach as Measured with Radioactive Phosphorus. J. Clin. Investigation, **28**:66–72, 1949.
19. Williams, H. H.; Kaucher, M.; Richards, A. J.; Moyer, E. Z.; and Sharpless, G. R. The Lipid Partition of Isolated Cell Nuclei of Dog and Rat Livers. J. Biol. Chem., **160**:227–32, 1945.

A Comparison of Nitrogen Content of Lymph Nodes from Cancer and Noncancer Patients*

JEANNE C. BATEMAN, CALVIN T. KLOPP, AND ANNE MENDELSON

(Departments of Medicine and Surgery and the Cancer Clinic of the George Washington University School of Medicine, Washington, D.C.)

Cancer patients, even when maintained in positive nitrogen balance, may show progressive decrease in serum protein and lack of clinical improvement (3). When weight gain occurs in such individuals, it does not account for all nitrogen retained (1). Increase in nitrogen content as well as hyperplasia of lymphatic tissue has been observed in mice bearing transplanted sarcomas and adenocarcinomas (2). Conversely, lymph nodes of tumor-bearing rats (Walker carcinoma 256) were found to contain less nitrogen than did the same tissues from pair-fed controls (5). This study was undertaken in an attempt to determine whether lymphatic tissue in human cancer patients acts as a possible source of nitrogen or competes with the growing tumor in consumption of nitrogen.

MATERIALS AND METHODS

Lymph nodes were obtained from cancer and noncancer patients, either at operation or post mortem examination. The nodes were immediately dissected free of fat and divided into two portions. One portion of each node was carefully weighed and then dissolved in equal parts of nitrogen-free sulfuric acid and water. A micro-Kjeldahl Nesslerization method was used for nitrogen determination, and readings were made on a Coleman junior spectrophotometer. The second portion was fixed in 10 per cent formalin, imbedded in paraffin, cut, and stained routinely with hematoxylin and eosin. Pathological examination was done to verify the type of tissue and to determine the presence or absence of cancer. Nodes showing fatty replacement on microscopic examination were not included in this study.

A limited number of blocks of tissue from various organs (liver, spleen, kidney, lung, and thyroid), as well as from tumors, was obtained at post mortem examinations. These were analyzed for nitrogen content by the same method employed in the examination of lymph nodes.

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RESULTS

Two hundred and ten nodes were obtained from 41 patients (28 surgical and 13 post mortem). Sex and age distribution were as follows:

Type case	Males	Females	Age range	Average age
Cancer	9	19	26–73 years	51.5 years
Noncancer	8	5	23–83 "	51.1 "

The diagnoses were as follows:

	Cancer cases			Noncancer	
	Surgical	Post mortem		Surgical	Post mortem
Breast	10	1	Heart disease		4
Skin	1		Nephritis		2
Tongue	2		Adenofibroma of breast	1	
Mouth	1		Intracranial hemorrhage		1
Metastatic to neck*	1		Peptic ulcer		1
Bronchus		1	Saphenous vein ligation	3	
Cervix		1	Intestinal perforation		1
Vulva	1				
Stomach	1				
Duodenum	1				
Colon	4		Total	4	9
Rectum	2				
Thyroid	1				
Total	25	3			

* Primary site unknown.

Nodes were classified in three groups: (*a*) nodes from noncancer patients, (*b*) nodes from cancer patients which on pathological examination showed the presence of neoplastic cells, and (*c*) nodes from cancer patients which on pathological examination were free of neoplastic cells. The range of nitrogen content, as well as the average for each group, is given in Table 1. The range is

TABLE 1

NITROGEN CONTENT OF NODES FROM CANCER AND NONCANCER PATIENTS

Type case	No. nodes	Nitrogen (gm/100 gm) Range	Av.
Noncancer	59	0.98–3.19	1.92
Cancer			
Neg. nodes	119	0.92–3.09	2.04
Pos. nodes	32	1.42–2.65	2.09

similar in nodes from noncancer patients and in noncancerous nodes from cancer patients and overlaps at both ends the range of nitrogen content of cancer nodes. There is no significant difference between the averages of the three groups at the 0.05 level of probability.

The nodes were further divided according to site as well as source. The range and average nitrogen content of each group are given in Table 2.

TABLE 2

NITROGEN CONTENT OF NODES GROUPED ACCORDING TO SITE

Site	Source	No. nodes	Nitrogen (gm/100 gm) Range	Av.
Axillary	Non-ca. pts.*	16	1.52–3.07	1.97
"	Ca. pts. neg. nodes	54	1.32–2.93	2.08
"	Ca. pts. pos. nodes	14	1.42–2.55	2.00
Cervical	Non-ca. pts.	2	2.50–2.67	2.58
"	Ca. pts. neg. nodes	26	1.35–3.09	2.27
"	Ca. pts. pos. nodes	2	2.40–2.46	2.43
Hilar	Non-ca. pts.	12	1.61–2.15	1.96
"	Ca. pts. pos. nodes	2	2.22–2.40	2.31
Inguinal	Non-ca. pts.	8	0.98–2.14	1.68
"	Ca. pts. neg. nodes	8	1.43–2.56	1.80
"	Ca. pts. pos. nodes	1		1.68
Mesenteric	Non-ca. pts.	14	1.27–3.19	1.91
"	Ca. pts. neg. nodes	28	0.92–2.35	1.87
"	Ca. pts. pos. nodes	5	1.45–2.65	2.22
Periaortic	Non-ca. pts.	7	1.61–2.30	1.86
"	Ca. pts. neg. nodes	3	1.67–1.83	1.72
"	Ca. pts. pos. nodes	5	1.85–2.19	2.04

* Pts. = patients.

The average nitrogen content was highest in nodes from the cervical region in all groups and lowest in nodes from the inguinal region, except for the periaortic negative nodes from cancer patients. When these statistics were analyzed, more variation was found among the different sites than should have been expected from the variation that existed within any one group. As a result, it can be said that there was a significant difference in nitrogen content between nodes from various sites.

In an attempt to correlate tumor size with total number of regional nodes, as well as percentage of metastatic nodes, nine cases of breast cancer are compared (Table 3).

The tumor specimens as well as the regional lymph nodes were obtained at surgery, since all the patients had radical mastectomies. None of these patients presented the extreme cachexia and malnutrition so frequently seen in far advanced neoplastic disease, so that this was a roughly homogeneous group as to site of disease and state of nutrition. As can be seen there was much greater variance in percentage of positive nodes than there was in total number of regional lymph nodes present. The size of the tumor did not seem to be related to either the total number or per cent positive regional lymph nodes present.

Nitrogen content of blocks of tissues from various sites from cancer and noncancer patients showed no appreciable difference, although the total number of specimens analyzed is small. The type of tissue, number of specimens, range of nitrogen, and average nitrogen content are given in Table 4. Tumor metastases in liver contained less

TABLE 3

SIZE OF TUMOR AND NO. OF REGIONAL LYMPH NODES IN NINE CASES OF RADICAL MASTECTOMY

Case	Tumor size (largest diameter) (cm.)	Regional lymph nodes Total no.	Metastatic no.	Per cent
CB	3.0	21	5	24
MB	4.0	24	1	4
AB	3.0	32	4	13
EG	5.0	8	1	12
EM	3.5	23	13	57
AH	2.5	15	10	67
RG	7.0	23	0	0
MA	3.0	15	0	0
JA	1.0	20	0	0

TABLE 4

NITROGEN CONTENTS OF BLOCKS OF TISSUE OTHER THAN LYMPH NODES

Tissue	No. specimens	Nitrogen (gm/100 gm) Range	Av.
Liver	10	2.16*–3.57	2.99†
Kidney	4	2.10 –2.67	2.38
Spleen	3	2.64 –3.28	3.29
Lung	3	2.02 –2.59	2.29
Muscle	2	2.46 –3.43	2.94
Thyroid	2	3.12 –3.29	3.20
Fat conn. tissue	2	0.40 –0.77	0.59
Tumor	7	2.26 –3.72	2.84

* This specimen was obtained from a liver showing gross fatty change.

† Does not include single specimen from fatty liver.

nitrogen in two specimens and more nitrogen in one specimen than adjacent hepatic tissue

DISCUSSION

In a careful study of the parametrium in 27 cases of carcinoma of the cervix, Sampson (4) found not only newly formed nodes projecting into lymph channels to which cancer could metastasize but also newly formed lymph nodes which apparently bore no relation to the lymph channels and in which cancer metastases were never seen. That growing tumor is associated with lymph node hyperplasia, even in the absence of metastatic involvement, was particularly apparent in the cases of carcinoma of the breast observed in

this study. Only a few small axillary nodes were obtainable from noncancer patients. In the presence of malignant neoplasm of the breast, large nodes were found in which no neoplastic cells were seen microscopically, and such cancer-free nodes greatly outnumbered those in which metastatic cancer was found.

Although serial sections were not done and the presence of a few neoplastic cells in some of these nodes may have been missed, the general increase in lymphoid tissue in the presence of cancer was striking. The masses of hyperplastic lymphoid tissue were found frequently as cords or even flat sheets between fat lobules. Occasionally in section a fat lobule was revealed, sheathed in an envelope of lymphoid tissue. Homburger (2) observed enlargement of lymph nodes, which was due to simple hyperplasia in mice bearing transplanted tumors. This increase in weight of lymphoid tissue was accompanied by a rise in nitrogen content. Sherman *et al.* (5) demonstrated a striking decrease in nitrogen content of lymph nodes, which in some cases was as great as 70–90 per cent in rats with transplanted Walker carcinoma 256, and postulated this tissue as one of the nitrogen sources for the growing tumor.

This study shows no significant difference in nitrogen content of nodes from noncancer and cancer patients. However, the increase in total lymphatic tissue in the region of the tumor suggests that, rather than being a source of nitrogen for the tumor, such tissue is a site of nitrogen shunt. The differences reported in lymphatic nitrogen in tumor-bearing animals may be attributed to species differences. Although we did not observe a significant absolute increase in lymph node nitrogen, the apparent increase in lymphatic tissue resembled that seen in tumor-bearing mice.

It is beyond the scope of this paper to discuss the mechanism of lymphoid hyperplasia associated with tumor growth. Willis (6) attributes these changes to mild bacterial infection in superficial growths, to degenerative changes in tumor or to blockage of ducts and retention of secretions in glandular organs. The observations in our study indicate that lymphoid tissue in human cancer patients does not act as a source of nitrogen and probably shares to a small extent with growing tumors in deviating nitrogen from its normal metabolic pathways.

SUMMARY

1. Two hundred and ten lymph nodes were obtained from 28 surgical and 13 post mortem examinations. These nodes were examined biochemically for nitrogen content and histologically for verification of type tissue and presence of cancer.

2. There was no significant difference between the nitrogen content of metastatic and nonmetastatic nodes from cancer patients and that of nodes obtained from noncancer patients.

3. A consistent hyperplasia of lymphoid tissue observed in cancer patients suggests that this tissue shares to some extent with growing tumor the role of depleting other body tissues of their nitrogen stores.

4. Significant differences of nitrogen content were observed in lymph nodes from various sites.

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REFERENCES

1. BATEMAN, J. C. Nitrogen Shift in Cancer. Arch. Int. Med., **86**:355–60, 1950.
2. HOMBURGER, F. Studies on Hypoproteinemia; III. Lymphoid Hyperplasia and Redistribution of Nitrogen Caused in Mice by Transplanted Tumors (Sarcoma 180 and Breast Adenocarcinoma E 0771). Science, **107**:648–49, 1948.
3. KOZOLL, D. D.; HOFFMAN, W. S.; and MEYER, K. A. Nitrogen Balance Studies on Surgical Patients Receiving Amino Acids. Arch. Surgery, **51**:59–68, 1945.
4. SAMPSON, J. A. A Careful Study of the Parametrium in 27 Cases of Carcinoma Cervicis Uteri and Its Clinical Significance. Am. J. Obst., **54**:433–64, 1906.
5. SHERMAN, C. D., Jr.; MORTON, J. J.; MIDER, G. B. Potential Sources of Tumor Nitrogen. Cancer Research, **10**:374–78, 1950.
6. WILLIS, R. A. Pathology of Tumours, p. 169. London: Butterworth & Co., Ltd., 1948.

The Distribution of Radioactivity in the Tissues of Mice Receiving Triphenylbromoethylene-Br^{82}*

GRAY H. TWOMBLY, ERWIN F. SCHOENEWALDT, AND DORIS MEISEL

(Department of Obstetrics and Gynecology, College of Physicians and Surgeons, Columbia University, New York, N.Y.)

In 1946 Daudel, Berger, Buu-Hoï, and Lacassagne (6) reported selective absorption of radioactive bromine by the ovaries of mice injected with the synthetic estrogen, triphenylbromoethylene. The estrogen had been made by a simple bromination of triphenylethylene with material obtained from one of the French atomic piles and had an activity of 100,000–500,000 counts per minute per milligram (2). When female mice belonging to the RIII strain were injected with this material dissolved in olive oil and were killed by decapitation at 12 hours, their ovaries were found to contain twice the quantity of radioactivity that occurred in the blood.

While these authors reported the use of both male and female mice in their investigations, they made no mention of measurements on the radioactivity of the testes. In fact, no tissue from the male animals was reported to have a higher concentration of radioactive bromine than did the blood, and all tissues gave much lower counts than was the case with the female mice.

In 1950 Daudel, Apelgot, Buu-Hoï, Costerousse, and Lacassagne (5) reported further experiments purporting to show concentration of 7 times the blood radioactivity in the ovaries, an eightfold concentration in the thyroid, a 48-fold concentration in the adrenal, and from 15 to 216 times the blood activity in the pituitary. These animals were killed at the end of 18 hours.

In 1940 Bonser and Robson (4) reported that triphenylethylene administered to Strong A male mice produced malignant Leydig-cell tumors. A second paper (3) by Bonser in 1944 showed that RIII males were also susceptible but not as susceptible as male Strong A mice. That tumor production was a function of estrogenic activity rather than specific chemical structure was suggested when Hooker, Gardner, and Pfeiffer (8) obtained similar results with estradiol benzoate and diethylstilbestrol. These observations were confirmed by Shimkin, Grady, and Andervont (10) with diethylstilbestrol and cholesterol pellets. Gardner (7) mentions that Leydig-cell tumors do not appear in C57 males treated with natural estrogens.

It was our purpose to verify the observations of the French investigators concerning the selective localization of the radioactive bromine of triphenylbromoethylene in mouse ovaries and to see whether a similar localization took place in the testes. We thought it might be possible to explain the observed strain differences in Leydig-cell tumor formation on chronic estrogenic stimulation by differences in uptake of radioactive triphenylbromoethylene.

MATERIALS AND METHODS

Radioactive bromine-82 was obtained by bombarding octyl bromide with slow neutrons in the Columbia cyclotron (Szilard-Chalmers reaction). The octyl bromide was extracted by repeated shakings with dilute aqueous sodium hydroxide. The sodium bromide so obtained was decomposed in a closed system by oxidizing it with manganese dioxide and sulfuric acid. With gentle heating the liberated bromine was carried over in a slow stream of nitrogen, through a drying trap, and into an iced receiver containing triphenylethylene in freshly distilled chloroform. The triphenylbromoethylene, obtained by evaporation of the chloroform and recrystallization from glacial acetic acid, appeared as white needles with a melting point of 114.5° C. to 115° C. It was freely soluble in sesame oil. The reaction is pictured in Chart 1.

The radioactive triphenylbromoethylene was obtained in small amounts, 3–5 mg. In each case it was dissolved in sesame oil and injected subcutaneously into groups of ten male mice, each mouse receiving 0.2 cc. of the solution containing 0.3–0.5 mg. of the synthetic estrogen or 700–2,500 counts/min of radioactive bromine. The mice were in lots of five each, strain A or RIII animals, which are known to develop Leydig-cell tumors, being compared simultaneously with C57 black or DBA animals. At the end of 22.5–23 hours they were killed and their organs dissected out carefully to avoid contamination. After weighing, the testes, adrenals, samples of liver and kidney, and the spleens were dissolved in 20 per cent

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KOH, the volume of each set of organs was made up to 5 cc., and their radioactivity was measured in a thin-window Geiger-Müller counter.

Subsequently, a similar procedure was carried out on two lots of animals of mixed sexes and strains: one male and two female C57 mice and two A females in the first lot; two female and two male C57 mice and two female and two male A mice in the second. In these animals, measurements of radioactivity of the ovaries, pituitaries, testes, liver, kidneys, and blood were made 6, 7, and 15 hours after injection. All these experiments were carried out with material of rather low specific activity.

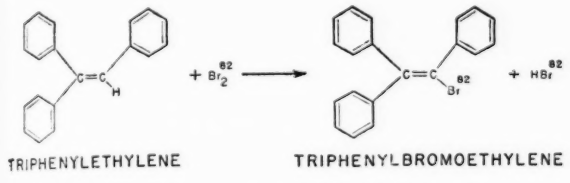

CHART 1.—Bromination of triphenylethylene

A final experiment designed to repeat the work of the French investigators as closely as possible was done in somewhat different fashion with material obtained from the Brookhaven pile. Two-hundred forty mg. of potassium bromate was bombarded with slow neutrons until it was estimated to have an activity of Br^{82} of 35 mc. The $KBrO_3$ was dissolved in 15 cc. of water and transferred to a small separatory funnel. Five cc. of purified chloroform was added, together with an oxidizing mixture (1 cc. saturated $KMnO_4$, 1 cc. concentrated HNO_3, and 2 cc. of water). The funnel was shaken vigorously for 30 seconds. The liberated bromine was dissolved in the chloroform by this procedure. It was withdrawn and chilled in an ice bath. Twenty mg. of triphenylethylene in 15 cc. of chloroform was added and the whole allowed to stand 2 hours in the cold. The chloroform was evaporated off, and the crystalline estrogen was dissolved in 2.5 cc. of olive oil. Each mouse received 0.2 cc. (2 mg. of triphenylbromoethylene) or 21,000,000 counts/min/mouse. Eight animals were injected: two male and two female C57 mice and two male and two female A mice. All animals were killed at the end of 18 hours, the organs removed, weighed, hydrolyzed, and their radioactivity determined.

RESULTS

Table 1 shows, in percentages of the total injected radioactivity, the amount recovered per milligram of the testes, adrenals, liver, kidney, and spleen. It is obvious that all organs lie within a narrow range of radioactivity. The least evidence of concentration in the testes of radioactive bromine of the administered estrogen was found in DBA males (0.0009 per cent/mg), the most in C57 males (0.0015 per cent/mg). The averages for animals susceptible to testicular tumor formation, A and RIII males, lie between these extremes: 0.0011 per cent/mg and 0.0012 per cent/mg. There is a suggestion in the figures that the radioactivity in the adrenal and kidneys may be slightly higher than in the spleen, but these differences are not considered significant.

TABLE 1

THE TISSUE LOCALIZATION OF RADIOACTIVITY FOLLOWING THE ADMINISTRATION OF TRIPHENYLBROMOETHYLENE-Br^{82}

Sex	No.	Testis	Adrenals	Liver	Kidney	Spleen
C57 male	10	0.0015	0.0010	0.0012	0.0015	0.0013
A "	"	0.0011	0.0021	0.0009	0.0014	0.0012
RIII "	"	0.0012	0.0016	0.0013	0.0018	0.0013
DBA "	"	0.0009	0.0010	0.0009	0.0010	0.0008

Percent of total injected radioactivity per milligram wet weight at 22–23 hours.

TABLE 2

THE TISSUE LOCALIZATION OF RADIOACTIVITY FOLLOWING THE ADMINISTRATION OF TRIPHENYLBROMOETHYLENE-Br^{82}

$$\text{Specific activity} = \frac{\text{counts/sec/mg tissue}}{\text{counts/sec/mg blood}}$$

	Testes or female organs	Pituitary	Kidneys	Adrenals	Liver
C57 mice:					
male	0.87	0.57	8.07	0.75	0.47
male	0.50	0.24	4.5	0.44	0.32
female	0.93*	0.24	7.47	0.65	0.35
female	1.88	0.47	8.48	0.97	0.44
Av.	0.68 (testes) 1.40 (♀ organs)	0.38	7.13	0.70	0.39
A mice:					
male	0.56	0.28	5.2	0.38	0.30
male	0.69	0.24	5.5	0.49	0.32
female	1.57	0.20	6.8		
female	0.77		7.0	0.61	0.34
Av.	0.62 (testes) 1.17 (♀ organs)	0.24	6.1	0.49	0.32
Data of Daudel *et al.*:					
Av. ovaries:	7.0	121	0.70	48	0.65

In Table 2, based on the final experiment, the figures are expressed in a slightly different fashion, to conform with the published results of Daudel *et al.*, whose findings are given at the bottom of the table for comparison. In this table the figures indicate the ratio of radioactivity in gonads, pituitary, kidneys, adrenals, and liver to that found in the blood. The testes contain one-half to three-quarters as much radioactivity as the blood, the ovaries three-quarters to almost twice that of blood, while in the pituitaries (in marked contrast to the findings of the French workers) the radioactivity averages only about one-third that of

blood. The values for liver are similar and those for adrenals a little higher. The radioactivity 18 hours after the administration of triphenylbromoethylene-Br^{82} was concentrated in the kidneys, with specific activities 5–8 times that of blood. We interpret this to mean that these organs are actively excreting the labeled estrogen.

DISCUSSION

While the present report must be considered a negative experiment, we consider it an important and significant one. Were selective absorption of a compound in any high ratio to be incontestably demonstrated, the means might be at hand for the easy destruction of organs showing such selectivity by the use of a radioactively labeled chemical. The literature pertaining to this question is meager. We have been unable to show any concentration of radioactive dibromoestrone in the organs of men or animals (11, 12), nor does radioactive stilbestrol seem to appear in significantly higher concentrations in the endocrine glands, uteri, or mammary glands of mice, dogs, or rabbits (13). Slight concentration of estradiol labeled with iodine-131 is reported by Albert, Heard, Leblond, and Saffran (1) in the breast, but there is some question as to how it got there. Possibly, subcutaneous administration may have had something to do with these findings.

Obviously, the measuring of a concentration of a synthetic estrogen in the ovaries of 2–13 times that found in the blood and in the pituitary up to 344 times that found in the blood, if true, would constitute a major discovery. Paterson, Gilbert, and Gallagher (9) attempted to confirm these observations for the ovary in 1948 but were unsuccessful. We regret that our findings have also been negative, for the selective absorption of triphenylbromoethylene by the pituitary—if it occurred—would constitute such a useful tool and point a hopeful finger toward other similar valuable methods of selective irradiation.

SUMMARY

1. Radioactive triphenylbromoethylene can be prepared with ease and with a high specific activity. Other investigators have shown that this compound is a potent synthetic estrogen, the brominated form being more estrogenic than triphenylethylene itself.

2. Because triphenylethylene on prolonged administration will cause the formation of Leydig-cell tumors in the testes of male mice of the A, JK, C, and RIII strains and will not do so in other strains such as C57 black, it was thought worthwhile to determine whether this difference in susceptibility to tumor formation could be related to a differing specific affinity of testicular tissues in various strains for the synthetic estrogen. Our observations that the radioactivity of the testes of four mouse strains, C57, A, RIII, and DBA, do not differ significantly after the injection of radioactive triphenylbromoethylene suggest that there is no selective absorption of this material by testicular tissue nor any difference in its local concentration in mice varying in their susceptibility to testicular tumor formation.

3. Repetition of the experiments of workers at the Curie Institute fails to confirm their observations that triphenylbromoethylene is concentrated to a high degree in the pituitary and adrenal glands.

REFERENCES

1. Albert, S.; Heard, R. D. H.; Leblond, C. P.; and Saffran, J. Distribution and Metabolism of Iodo-α-Estradiol Labeled with Radioactive Iodine. J. Biol. Chem., **177**:247–66, 1949.
2. Berger, M.; Daudel, P.; and Buu-Hoï, N. P. Le métabolisme d'une substance oestrogène étudié au moyen du brome radioactif. J. de radiol. et d'électrol., **28**:238–39, 1947.
3. Bonser, G. M. Mammary and Testicular Tumours in Male Mice of Various Strains Following Oestrogen Treatment. J. Path. & Bact., **56**:15–26, 1944.
4. Bonser, G. M., and Robson, J. M. The Effects of Prolonged Oestrogen Administration upon Male Mice of Various Strains; Development of Testicular Tumours in the Strong A Strain. J. Path. & Bact., **51**:9–22, 1940.
5. Daudel, P.; Apelgot, S.; Buu-Hoï, N.P.; Costerousse, O.; and Lacassagne, A. Métabolisme du triphenylethylene brome, étudié chez la souris, par la méthode des indicateurs radioactifs. Bull. Soc. chim. biol., **32**:264–67, 1950.
6. Daudel, P.; Daudel, R.; Berger, M.; Buu-Hoï, N. P.; and Lacassagne, A. Étude du métabolisme d'un oestrogène par la méthode des indicateurs radioactifs. Experientia, **2**:107–8, 1946.
7. Gardner, W. U. Testicular Tumors in Mice of Several Strains Receiving Triphenylethylene. Cancer Research, **3**:92–99, 1943.
8. Hooker, C. W.; Gardner, W. U.; and Pfeiffer, C. A. Testicular Tumors in Mice Receiving Estrogens. J.A.M.A., **115**:443–45, 1940.
9. Paterson, E.; Gilbert, C. W.; and Gallagher, U. M. Metabolism of the Oestrogen Triphenylbromoethylene. Nature, **163**:801, 1949.
10. Shimkin, M. B.; Grady, H. G.; and Andervont, H. B. Induction of Testicular Tumors and Other Effects of Stilbestrol-Cholesterol Pellets in Strain C Mice. J. Nat. Cancer Inst., **2**:65–80, 1941.
11. Twombly, G. H.; McClintock, L.; and Engelman, M. Tissue Localization and Excretion Routes of Radioactive Dibromestrone. Am. J. Obst. & Gynec., **56**:260–68, 1948.
12. Twombly, G. H., and Schoenewaldt, E. F. The Metabolism of Radioactive Dibromoestrone in Man. Cancer, **3**: 601–7, 1950.
13. ———. Tissue Localization and Excretion Routes of Radioactive Diethylstilbestrol. *Ibid.*, **4**:296–302, 1951.

Studies on the Metabolism, Distribution, and Excretion of 2-*p*-Toluenesulfonamidofluorene-S^{35} in the Rat*†

FRANCIS E. RAY AND MARY F. ARGUS‡

(*Cancer Research Laboratory, University of Florida, Gainesville, Fla.*)

That 2-acetylaminofluorene (AAF) is a potent carcinogen when fed to rats was first established in 1941 by Wilson, DeEds, and Cox (17). Since then, it has definitely been shown that AAF is capable of producing a variety of tumors in the animal body at numerous sites generally far removed from the point of application (2, 3, 8, 18).

Recently, the preparation (12) and investigation (9) of the radioactive forms, 2-acetylaminofluorene-9-C^{14} and 2-acetylaminofluorene-ω-C^{14}, have greatly extended our knowledge of the metabolism of AAF. Among the conclusions established is that 6 per cent of the C^{14} ingested as 2-acetylaminofluorene-ω-C^{14} is expelled as respiratory CO_2 within 6 hours. This shows that deacetylation of AAF in the animal body can take place.

The observation by Morris *et al.*, (7) that 2-aminofluorene (AF) is also carcinogenic raised the question whether conversion to the free amine is a necessary prelude to tumor incidence.

In general, the acetyl group is readily removed from aromatic amines. If the acetyl group of AAF could be replaced with groups which are less readily hydrolyzed or not removed at all, the study of such derivatives would throw light on the subject. It was predicted that, if conversion to the free amine is a necessary step, the carcinogenicity of such derivatives would vary directly with the ease of removal of these groups. The benzoyl group is hydrolyzed with difficulty, while no enzyme is known to remove the tosyl group ($CH_3C_6H_4SO_2$-). Accordingly, 2-benzoylaminofluorene and 2-*p*-toluenesulfonamidofluorene were prepared.

In harmony with our predictions, Morris[1] reports that the 2-benzoyl compound is considerably less active than AAF, while the tosyl derivative is noncarcinogenic.

Does the difficulty of hydrolysis of the tosyl group retard the absorption and metabolism of this derivative to such an extent that it is eliminated unchanged from the animal body? To endeavor to answer this question we administered 2-*p*-toluenesulfonamidofluorene-S^{35} to rats and traced the compound by the radioactivity[2] in organs and excreta. We also examined the urine and feces to determine if the compound had undergone metabolic change.

MATERIALS AND METHODS

Four-month-old Sprague-Dawley (Holtzman) strain rats with an average body weight of 350 gm. were employed. The 2-*p*-toluenesulfonamidofluorene-S^{35} ($TS^{35}AF$) was prepared[3] by the method of Cambell, Anderson, and Gilmore (4). The radioactive sulfonyl chloride used in this procedure was prepared by the method of Ray and Soffer (13). The $TS^{35}AF$ (m.p. 157°–159° for the first and second experiments, m.p. 160°–161° for the third experiment) was used in the form of a coconut oil solution,[4] 10 mg. $TS^{35}AF$/ml oil. Administration was by stomach tube. Each rat was fasted 24 hours prior to receiving a 2-ml. dose of the $TS^{35}AF$–coconut oil solution and then permitted unlimited food (Purina Dog Chow) through the remainder of the experiment. The animals were allowed water at all times.

Following treatment, each rat was placed in a specially constructed cage which facilitated the separation and collection of urine and feces samples and prevented the urine from coming in contact with the feces. The animals were kept in an air-conditioned room at 24° C., except in the

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‡ With the technical assistance of Gilbert Bergquist and Shyne Marley.

[1] H. P. Morris, private communications.

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[2] Radioactive sulfur in the form of $H_2S^{35}O_4$ was obtained from the Oak Ridge National Laboratory on allotment by the Atomic Energy Commission.

[3] We are indebted to Dr. O. H. Borum for the preparation of the 2-*p*-toluenesulfonamidofluorene-S^{35} used in this study.

[4] We wish to thank Proctor & Gamble Co. for the coconut oil used in this investigation.

case of the third experiment where unavoidable conditions necessitated keeping the animal at a room temperature of about 27° C. Feces samples were weighed immediately following collection and then air-dried.

In the first experiment urine and feces samples collected for a 54-hour period were studied. The animal was deprived of food at 8 A.M., and the $TS^{35}AF$ solution was administered at 8 A.M. the following day. The animal was under observation between the 1st and 12th hours, the 24th and 36th hours, and the 48th and 54th hours, during which intervals urine and feces were measured immediately on excretion. Collective samples for the 12-hour periods between the 12th and 24th hours and the 36th and 48th hours were made.

The animal of the second experiment was treated at 8 P.M., and urine and feces samples were collected as described above, but for the 12-hour periods alternate to those of the first run. At the 66th hour this rat was anesthesized with ether and the pericardial cavity opened. The heart was exposed, the left ventricle was slit with scissors, and the heart was allowed to pump the blood into a graduated centrifuge tube containing 1 ml. of 1.1 per cent sodium oxalate. The 5.7 ml. of blood collected was centrifuged to separate formed elements from plasma. The total blood volume was calculated on the basis of 6.7 ml/100 gm body weight (6). The contents of the stomach, small intestine, and large intestine, including the cecum, were obtained quantitatively, weighed, and air-dried.

During the third run, individual urine and feces samples were collected for a 24-hour period. At the 24th hour the blood and contents of the stomach, small intestine, and large intestine were removed as before. The liver and kidneys of this animal were also removed and weighed.

The various samples and organs were then prepared for radioactivity determination in the following manner. After drying, the samples of feces and stomach and intestinal contents were ground to a fine powder in a mortar. A suspension of each in 1 per cent sodium hydroxide (containing 5 drops of the wetting agent, Tergitol, to each 25 ml. of solution) was then prepared by mixing in a Waring Blendor for 30 minutes. Fifty ml. of sodium hydroxide solution was used for each 10 gm. of sample. A few drops of capryl alcohol were added to prevent foaming. The suspension was allowed to stand 24 hours at 5° C. The mixture was then brought to room temperature and again agitated in a Waring Blendor. A 1-ml. portion was placed on a shallow aluminum planchet and allowed to dry at room temperature. The liver and kidneys were prepared one-half as concentrated by macerating the tissue in a Waring Blendor in the presence of 1 per cent sodium hydroxide solution (25 ml. of solution to 10 gm. of tissue). Planchets were prepared from this suspension as described above. One-ml. portions of the urine, blood plasma, and blood cell samples were plated directly on planchets.

Radioactivity measurements were made in an internal-type counter[5] with an efficiency of 45 per cent. The $TS^{35}AF$ had an activity of 49,700 counts/min/mg. Each sample was counted for three 10-minute intervals, and the net counts per minute above background were recorded for each sample.

The concentration of $TS^{35}AF$ in these samples was determined by direct comparison with standard planchets prepared in the same manner and containing known concentrations of $TS^{35}AF$. Feces standards were used for determining the feces, and stomach and intestinal contents; liver standards for the liver, kidneys, and blood cells; and urine standards for the urine and blood plasma samples. In order to eliminate correction for the decay of S^{35}, the standards were counted on the same day as the samples.

The procedure used for the estimation of AF in the urine was the photometric method of Westfall and Morris (16), modified so as to permit estimation of both conjugated and free AF. Urine obtained from an animal of the same strain and age to which 2 ml. of coconut oil had been administered was treated in the same manner as the experimental samples and was used as a blank in the colorimetric analysis. Standards were prepared containing varying known concentrations of freshly prepared AF (m.p. 127°) per milliliter of urine. Urine for the standards was obtained under the same conditions from untreated animals. The instrument employed for the photometric analysis was a Beckman quartz spectrophotometer, model DU.

Material for the carrier experiments was obtained in the following manner. The suspension of feces (experiment 3) in 1 per cent sodium hydroxide was evaporated in air to dryness. A sample of TSAF which had previously been allowed to stand a comparable period of time in 1 per cent sodium hydroxide was found to be unaffected. The dried feces was then extracted with 10-ml. portions of acetone until no activity was detectable in the residue. Ten mg. of the active residue obtained by evaporation of the acetone filtrates was successively recrystallized 8 times with 40 mg.

[5] Q-gas chamber and Nuclear Instrument and Chemical Corporation Scaler, Unit Model 162.

of carrier TSAF from 70 per cent ethanol. The active material from the urine was obtained by evaporating the pooled urine samples to dryness in air and extracting with three 10-ml. portions of acetone and two 10-ml. portions of 70 per cent ethanol. This was recrystallized with carrier TSAF, as described for the material obtained from the feces. Carrier experiments of the active material from the urine with sodium *p*-toluenesulfonate were also carried out. The carrier compound and active material were first refluxed together in ethanol for 2 hours to bring about ion exchange. The recrystallizing medium for this compound was 95 per cent ethanol. To test for radioactivity in the inorganic sulfate, benzidine sulfate was precipitated from the pooled urine samples.

RESULTS AND DISCUSSION

The distribution of radioactivity in the organs and excreta of rats following administration of TS^{35}AF is given in Table 1. The first animal was observed for 54 hours, and it may be seen that, while 67 per cent of the material was excreted in the feces, only 0.5 per cent appeared in the urine.

The time for the second animal was extended to 66 hours. At the end of this time no radioactivity was detectable in the blood. There was a small amount of the material in the intestines, but none remained in the stomach. Over 90 per cent had been eliminated in the feces and 0.4 per cent in the urine. A total of 92.4 per cent of the sulfur was accounted for.

Because the material had been completely eliminated from the blood at 66 hours, we examined the distribution of TS^{35}AF at an intermediate time—24 hours. In this third animal, a little less than 0.3 per cent was eliminated in the urine and 63.7 per cent in the feces. Two per cent remained in the stomach, the small intestine contained 8 per cent, and the large intestine 30 per cent. The liver had about 1 per cent and the kidney less than 0.1 per cent. The blood plasma now showed definite evidence of a low concentration of the compound, with none in the blood cells. This agrees with the findings of Morris and Westfall (10), since all the AAF detectable in the blood by diazotization was found in the plasma. The total S^{35} activity accounted for in the third experiment was 106.0 per cent. This error on the positive side is just about the same as that of the preceding sample on the negative side.

The peak in the elimination in the feces comes at 16 hours after administration. The amounts of radioactive material found in the urine at intervals following feeding of TS^{35}AF reach a maximum at about 6 hours and then gradually drop off. Morris and Westfall (11) found that the peak in the concentration of diazotizable AAF in the rat urine came at the 4–6-hour period.

In Chart 1 a comparison of the distribution of TS^{35}AF and AAF in the rat is made. The data for AAF was determined on the basis of diazotizable nitrogen (10, 11), on the activity of the C^{14}-labeled compound (9), and on mass spectrographic analysis of AAF-N^{15} (5).

After 16 hours Morris and Westfall (10) found 7–25 per cent of the ingested dose of AAF still remaining in the stomach. At 24 hours we found about 2 per cent TSAF in the stomach, but none remained in this organ at 66 hours.

Using a dose of 16 mg AAF/100 gm of rat, which was comparable to our dosage of TSAF, Morris and Westfall (11) found that 28–45 per cent was eliminated in the urine, as compared with 0.5 per cent of our material, at 24 hours. Using the radioactive AAF, the only results are available for a 6-hour period, and here about 6–7 per cent of the material was recovered in the urine. The greatest part of this compound, 75 per cent, was still in the stomach. Over 1 per cent of the AAF was

TABLE 1

DISTRIBUTION OF RADIOACTIVITY IN THE RAT FOLLOWING ORAL ADMINISTRATION OF 2-*p*-TOLUENESULFONAMIDOFLUORENE-S^{35}
(20 mg. TS^{35}AF in 2 ml. coconut oil)

	Rat no. 1, 54 hours		Rat no. 2, 66 hours		Rat no. 3, 24 hours	
	Mg. recovered	Per Cent recovered	Mg. recovered	Per cent recovered	Mg. recovered	Per cent recovered
Urine	0.1	0.5	0.1	0.4	0.06	0.3
Feces	13.4	67.1	18.1	90.6	12.7	63.7
Stomach contents			0.0	0.0	0.4	2.2
Small intestine contents			0.1	0.5	1.7	8.4
Large intestine contents			0.2	0.9	6.1	30.3
Blood plasma			0.0	0.0	0.01	0.06
Blood cells			0.0	0.0	0.0	0.0
Liver					0.2	1.0
Kidney					0.01	0.04
Total	13.5	67.6	18.5	92.4	21.2	106

found in the kidney, whereas less than 0.1 per cent of the tosyl compound was in this organ. This is to be expected, in view of the much higher proportion of AAF eliminated in the urine.

The carrier experiments carried out on the material eliminated by the intestine showed it to be unchanged TSAF. Examination of the material excreted in the urine, however, showed that it had been metabolized, the original compound not being present.

When this urine was subjected to the modified diazotization and coupling procedure (16), it was found that free AF was present as 0.48 per cent of the ingested dose of TSAF. It is thus evident that

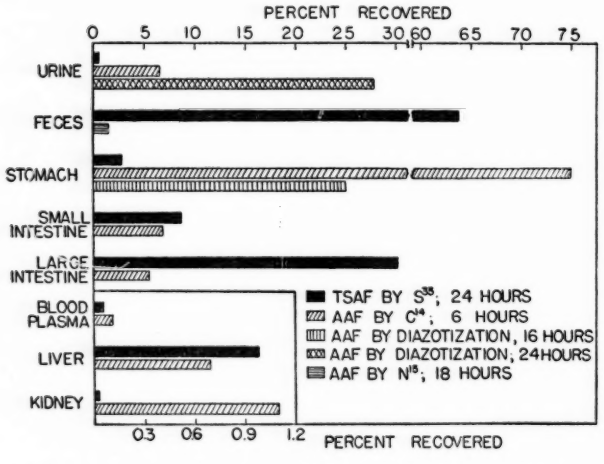

CHART 1.—A comparison of the distribution of TSAF and AAF in the organs and excreta of the rat. The scale for the blood plasma, liver, and kidney recovery has been proportionally magnified.

all the compound accounted for in the urine was in the completely hydrolyzed form.

In an attempt to determine what other metabolite of TSAF was present in the urine, the urinary inorganic sulfate was precipitated as benzidine sulfate. No radioactive inorganic sulfate was present.

The hydrolysis of TSAF would be expected to yield, in addition to AF, *p*-toluenesulfonate. Carrier experiments carried out with this compound failed to establish its presence. Either before or after hydrolysis, the *p*-toluenesulfonic acid moiety was metabolized. Oxidation of an aromatic methyl group to the carboxy acid is a known metabolic process. It is possible that the other metabolite of TSAF is *p*-sulfobenzoic acid.

Since these studies show that over 90 per cent of the ingested TSAF is excreted unchanged in the feces, it may be suggested that the compound is noncarcinogenic because it is not absorbed in sufficient concentrations by organs most likely to be attacked. The following facts, however, contradict this idea. The amount of radioactive AAF in the liver at 6 hours was 0.7 per cent, which is comparable with the TSAF value (1.0 per cent) at the end of 24 hours. After 6 hours, however, Morris and Westfall (10) found only 0.2 per cent of the AAF in the liver by diazotization. This difference in the two values for AAF is significant. In the first case, the AAF was determined in terms of the C^{14}. The second value is based on diazotizable AF available by extraction and hydrolysis. The fact that less is accounted for by the diazotization method indicates that a substantial portion of the AAF in the liver is in a modified form. Thus, the carcinogenicity of AAF in the liver is not the result of its concentration per se but is a consequence of the state of the compound in the liver. We find that the tosyl derivative is present in the liver in larger concentrations and for a longer period of time than AAF. If TSAF were capable of cancer production, it has sufficient contact with this organ to display this activity. One possible explanation for this lack of carcinogenesis is that the TSAF, unlike AAF, is a more stable compound and is not metabolized in the liver.

Another noteworthy result is that the concentrations of both TSAF and AAF in the blood plasma are equal. Morris and Westfall (10) report 100 μg AAF/100 ml blood plasma, 16 hours after ingestion. We found this same level (100 μg TSAF/100 ml blood plasma) even after 24 hours.

The question still remains whether AF is the primary carcinogen, and whether conversion of substantial amounts of a derivative to the free amine is a necessary prelude to carcinogenesis. An objection to this theory is the evidence that AAF is the primary carcinogen. It is known that a variety of aromatic amines are acetylated *in vivo*. In addition, Wilson, DeEds, and Cox (19) report that in the rat AF is a slightly slower-acting carcinogen than AAF, a fact which would not at first be expected if AAF is active only through conversion to AF. In view of these facts, then, AAF could be considered the primary carcinogen, no matter in what form the AF was supplied, provided that AF could be formed *in vivo* from the other derivatives. Yet even if this were the case, the necessity exists for the hydrolysis of a fluorene derivative to AF prior to acetylation.

On the other hand, Morris *et al.* (9) have shown that AAF undergoes deacetylation in the animal body. Also, the lower activity of AF compared to AAF in the rat is not too indicative of primary AAF activity. Animals ingesting 2-diacetylaminofluorene (di-AAF) develop tumors in a shorter time than is necessary for either AF or AAF (8).

It cannot be inferred, nevertheless, that di-AAF is the primary carcinogen! Moreover, the lower activity of AF in the rat may be due to solubility differences. Since AF is considerably more soluble than AAF (15), it may be excreted more quickly; hence, the tissues are not exposed as long as with AAF, which is excreted at a rate depending on its hydrolysis to AF. Furthermore, Wilson *et al.* (19) point out that in the mouse AF is possibly more active than AAF, since it shows a slightly shorter period of incubation.

From the evidence available at present, it does not seem possible to reach a conclusion in this matter. One definite way this could be settled, however, would be by feeding AF to dogs and determining if it has a carcinogenic effect. Allison *et al.* (1) have shown that AAF is carcinogenic in dogs. Acylase for acetylating aromatic amines is absent from dogs (14), but acetyl derivatives of aromatic amines can be deacetylated by oxidation. Therefore, AAF can go to AF, but AF cannot be converted to AAF. If AF proves carcinogenic in dog, it is unquestionably the primary carcinogen.

SUMMARY

The distribution of radioactivity was studied in the organs and excreta of the rat at 24-, 54-, and 66-hour intervals following oral administration of a single dose of noncarcinogenic 2-*p*-toluenesulfonamidofluorene-S^{35}.

The amount found in the liver (1.0 per cent) and in the blood (100 μg/100 ml plasma) was comparable to the amounts of 2-acetylaminofluorene found in similar experiments. The noncarcinogenicity of 2-*p*-toluenesulfonamidofluorene, therefore, is not caused by insufficient concentration of the compound in the liver (a predominant site for 2-acetylaminofluorene-induced tumors) or in the blood. Over 90 per cent of the ingested dose was found to be eliminated unchanged through the gastrointestinal tract. Only 0.5 per cent of the radioactivity was found in the urine. All the compound accounted for in the urine was in the form of 2-aminofluorene. This minute quantity of 2-*p*-toluenesulfonamidofluorene which undergoes metabolism contrasts with the relatively large amount, approximately 30 per cent, of the carcinogenic 2-acetylaminofluorene appearing in the urine.

REFERENCES

1. ALLISON, J. B.; WASE, A. W.; LEATHEM, J. H.; and WAINIO, W. W. Some Effects of 2-Acetylaminofluorene on the Dog. Cancer Research, **10**:266–71, 1950.
2. BIELSCHOWSKY, F. Distant Tumors Produced by 2-Amino and 2-Acetyl-aminofluorene. Brit. J. Exper. Path., **25**:1–4, 1944.
3. ———. The Carcinogenic Action of 2-Acetylaminofluorene and Related Compounds. Brit. M. Bull., **4**:382–85, 1947.
4. CAMBELL, N.; ANDERSON, W.; and GILMORE, J. Structure of Aromatic Compounds. J. Chem. Soc., pp. 446–51, 1940.
5. DYER, H. M.; ROSS, H. E.; and MORRIS, H. P. A Comparison of the Distribution of Isotopic Nitrogen and Diazotizable Nitrogen of N^{15}-2-acetylaminofluorene in the Rat. Cancer Research, **11**:244–45, 1951.
6. FARRIS, E. J., and GRIFFITH, J. Q. (eds.). The Rat in Laboratory Investigations, p. 413. Philadelphia: J. B. Lippincott Company, 1949.
7. MORRIS, H. P.; DUBNIK, C. S.; DUNN, T. B.; and JOHNSON, J. M. Tumors Produced in Rats after Ingestion or Painting of 2-Nitro, 2-Amino, N-acetyl-2-amino, and N-diacetyl-2-aminofluorene. Cancer Research, **7**:730–31, 1947.
8. MORRIS, H. P.; DUBNIK, C. S.; and JOHNSON, J. M. Studies of the Carcinogenic Action in the Rat of 2-Nitro-, 2-Amino-, 2-Acetyl-amino-, and 2-Diacetylaminofluorene after Ingestion and after Painting. J. Nat. Cancer Inst., **10**:1201–13, 1950.
9. MORRIS, H. P.; WEISBERGER, J. H.; and WEISBERGER, E. K. The Distribution of Radioactivity Following the Feeding of Carbon 14–labeled 2-Acetylaminofluorene to Rats. Cancer Research, **10**:620–24, 1950.
10. MORRIS, H. P., and WESTFALL, B. B. Distribution of N-acetyl-2-aminofluorene in the Rat Following a Single Feeding. J. Nat. Cancer Inst., **9**:149–54, 1948.
11. ———. Some Studies of the Excretion of Diazotizable Material after Feeding 2-Acetylaminofluorene to Rats. Cancer Research, **10**:506–9, 1950.
12. RAY, F. E., and GEISER, C. R. Synthesis of 2-Acetylaminofluorene-9-C^{14} and 2-Acetylaminofluorene-ω-C^{14}. Cancer Research, **10**:616–19, 1950.
13. RAY, F. E., and SOFFER, L. Compounds for Cancer Research. V. Radioactive Sulfonamides. J. Org. Chem., **15**: 1037–42, 1950.
14. STEKOL, J. A. Detoxication Mechanisms. Ann. Rev. Biochem., **10**:265–84, 1941.
15. WESTFALL, B. B. Estimation of 2-Aminofluorene and Related Compounds in Biological Material. J. Nat. Cancer Inst., **6**:23–29, 1945.
16. WESTFALL, B. B., and MORRIS, H. P. Photometric Estimation of N-acetyl-2-aminofluorene. J. Nat. Cancer Inst., **8**:17–22, 1947.
17. WILSON, R. H.; DEEDS, F.; and COX, A. J. The Toxicity and Carcinogenic Activity of 2-Acetaminofluorene. Cancer Research, **1**:595–608, 1941.
18. ———. Carcinogenic Activity of 2-Acetaminofluorene. II. Effects of Concentration and Duration of Exposure. *Ibid.*, **7**:444–49, 1947.
19. ———. The Carcinogenic Activity of 2-Acetaminofluorene. IV. Action of Related Compounds. *Ibid.*, pp. 453–58.

Rous Sarcoma in Folic Acid-deficient Chicks
Morphology and Bioassay*

E. Woll,† A. C. Dornbush, and P. A. Little

(Research Department of the Lederle Laboratories, American Cyanamid Co., Pearl River, N.Y., and the Departments of Pathology and Oncology, University of Vermont, Burlington, Vt.)

INTRODUCTION

Rous sarcoma in chicks which were on a folic acid-free diet, or which received certain of the folic acid antagonists in food or by parenteral injection, was either prevented or markedly inhibited (5). The general pathology of chicks (6) and rats (7) with diet- or antagonist-induced folic acid deficiency was then investigated. The chief changes were in the bone marrow and the gastrointestinal tract. In the marrow there was a transient maturation arrest of all blood cell elements at the level of the stem cells with megaloplasia. This was followed by a total and profound aplasia. In the intestine there was, initially, a marked hydropic swelling of the mucosal epithelium which involved both cytoplasm and nucleus (Figs. 1 and 2). This progressed to severe atrophy with associated ulceration and hemorrhage. The stomach invariably escaped this process. In the chicks the above changes were the same in the dietary or antagonist-produced deficiency, and recovery was seen when the deficient diet was supplemented with the vitamin.

The purpose of this investigation was: (*a*) to compare the microscopic anatomy of the control and the inhibited Rous sarcoma; (*b*) to compare this to the marrow and intestinal changes seen in chicks with folic acid deficiency; and (*c*) to measure, by means of a bioassay, the folic acid content of the tumor and of the host tissue in birds on control, folic acid-free, folic acid antagonist-containing diets and a folic acid-free diet supplemented with folic acid.

MATERIALS AND METHODS

A homogenized sample of Rous sarcoma cells was prepared by means of a Waring Blendor and Ten Brock grinder. A suspension containing approximately 10 mg. of tumor/0.25 ml of saline was then passed through a Berkefeld filter. One-half of 1 cc. of the filtrate was injected into the pectoral muscles of 120 1-day-old New Hampshire Red chicks. The down of the injection site had been removed previously. The birds of this series were divided into groups of 25–30 and were placed on the following regimes.

Group 1.—Control, a commercial baby chick feed.

Group 2.—A synthetic folic acid-free diet (its composition was described in a previous report [5]).

Group 3.—A commercial baby chick feed, as above, plus 80 mg. of 4-aminopteroylaspartic acid mixed into each kilogram of feed.

Group 4.—A synthetic diet, as above, plus a daily intraperitoneal injection of 100 μg. of folic acid in aqueous solution.

A second series of 80 birds, on the same dietary regimes, was divided into 2 sub-groups of 10 each. One sub-group was inoculated with the tumor filtrate as above. The other received an injection of 0.5 cc. of saline only.

Two to four birds from each of the groups in the first series were decapitated on the 2d, 3d, 4th, 7th, 10th, 15th, and 20th days of the experiment, and an autopsy was performed immediately. Multiple blocks of the tumor or of the injection site, as well as of the viscera, were taken for microscopic examination. The tissue was fixed in formaldehyde-Zenker solution, imbedded in paraffin, and stained with hematoxylin and eosin, phosphotungstic acid-hematoxylin, and Schorr's modification of Masson's trichrome stain.

All the animals of the second series were sacrificed on the fifteenth day of the experiment and were examined at autopsy immediately. The tumors were dissected out with care, to exclude nonsarcomatous tissue. The pectoral muscles of

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† Present address: College of Medicine, University of Vermont, Burlington, Vt.

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the saline-injected animals were also excised. The yield from each sub-group was pooled and stored in separate containers in dry ice until used. Weighed equal suspensions of the material were then assayed, with *Lactobacillus casei* as the test organism and folic acid as the standard. The method was that described by Teply and Elvehjem (4), except that Norit-treated peptone and DL-alanine were omitted from the basal media.

RESULTS

SERIES 1

Group 1 (commercial feed).—Every bird showed a rapidly growing tumor with the characteristic infarction, hemorrhage, and myxomatous consistency. Microscopically, the typical cell was small, round, with a delicate, scant, homogeneous cytoplasm, and finely distributed nuclear chromatin (Fig. 3). The cells were arranged in syncytoid masses. Mitoses were infrequent. Little or no reactive change was seen at the margins of the growth. Microscopic pulmonary metastases were found frequently. These showed the same features as the primary tumors.

Group 2 (folic acid-free diet).—For the first 7 days, there was no gross evidence of tumor. However, multiple sections of the inoculation site, at many levels, did reveal minute microscopic foci of tumor as early as the 3d day. Later in the experiment (15–20 days), a rare, very small, pale yellow, firm tumor nodule was detected. This is at variance with a previous observation (5), based on gross examination, that no tumor was found in chicks on a folic acid-free diet.

Microscopically, from the earliest evidence of tumor to the end of the experiment, a uniform pattern could be discerned. As compared to the control, the typical cell was much larger; it was round or polygonal. The cytoplasm was more abundant and dense. Centrally, it was coarsely granular, while the margin was vacuolated. The nucleus also was much larger. It was vesicular and contained a single dense nucleolus. Mitotic figures were seen with the same low frequency as in the controls. Phagocytosis by the tumor cells was common (Fig. 4). The older tumor (20 days) showed active proliferation of fibroblasts and capillaries. Collagen deposition was limited to the normal-appearing young fibroblasts. There was also a peripheral accumulation of lymphocytes. Unexpected were two instances of microscopic pulmonary metastasis. The same pattern as in the primary tumor was seen, except for the lack of reactive changes.

Group 3 (commercial feed with antagonist supplement).—The outstanding finding was the almost complete absence of tumor, grossly or microscopically. From the fifteenth day on, three instances of a single microscopic focus were found. The pattern was identical in every respect to that seen in the diet-deficient group.

Group 4 (folic acid-free diet plus folic acid by injection).—The tumor was recognized on inspection as early as 2 days after inoculation. Growth was distinctly more rapid and voluminous than in the control animals. The microscopic appearances were essentially identical to the controls, except in three animals sacrificed on the tenth, fifteenth, and twentieth days. In these there was little necrosis or hemorrhage in the tumor. It was firm and lobulated. The predominant histologic feature was compact fascicles of well preserved spindle cells with long delicate processes. The small round cells were also present but only in the few soft and partly liquefied areas.

It may be emphasized that the above features were not exclusive in every detail. Individual cells, representative of each of the types described, could be found in any of the groups studied. On the other hand, the predominance of one pattern was strikingly constant for each group.

A comparison of the cytologic alterations in the bone marrow and intestine of folic acid-deficient animals with those of chicks with inhibited Rous sarcoma revealed the following common features: a marked hydropic swelling of the cell (nucleus and cytoplasm), loss of chromatin detail, and arrest of growth. As is well known, this alteration, as judged by conventional stains, is not specific but is rather a common morphologic characteristic of various cellular disturbances. Levine (1), in his thorough investigation of the cytology of Rous sarcoma, described and illustrated the above noted large cells of slowly growing tumors in naturally resistant birds. Similar observations were made by Rous and Murphy (7). We have observed the same phenomenon in a few of a group of older birds, but never in baby chicks.

SERIES II

Bioassay.—The findings of this procedure are shown in Chart 1. It is to be noted that the tumor contains substantially more folic acid than the pectoral muscle—the tumor site. In the presence of dietary folic acid deficiency, the concentration in the muscle is markedly reduced, while the high level in the tumor is maintained. On a folic acid–supplemented diet, there is increased concentration in both muscle and tumor. However, the amount in the tumor is still relatively higher. In the presence of an antagonist the bioassay fails as a measure of folic acid content.

These findings are in accord with the observations of Pollack (2), who, in a bioassay of a large series of human, rat, and mouse tumors, found the concentration of folic acid to be high in contrast to the other B vitamins.

BIOASSAY OF FOLIC ACID CONTENT

DIET	FOLIC ACID MICROGRAMS / GM.	
	MUSCLE	TUMOR
COMMERCIAL MASH	0.0123	0.0250
FOLIC ACID-FREE	0.0030	0.0244
FOLIC ACID-FREE PLUS F.A. 10 MGM/KGM/DAY	0.0242	0.0409
COMMERCIAL MASH PLUS 80 MGM/KGM./DAY 4-AMINO-PTEROYL ASPARTIC ACID	INHIBITED	INHIBITED

CHART 1.—Folic acid content of Rous sarcoma tissue and of the pectoral muscles (the tumor site), as determined by bioassay, in chicks maintained on control, folic acid-free, folic acid antagonist-containing and folic acid-free diet supplemented with folic acid.

SUMMARY AND CONCLUSIONS

1. The morphology of Rous sarcoma in chicks made folic acid-deficient by means of diet or antagonist was compared to the alterations in the bone marrow and gastrointestinal tract.

2. Similar comparisons were made with birds on a control diet, as well as on a folic acid–free diet supplemented with folic acid.

3. The folic acid content of the tumor tissue and of the pectoral muscle (the tumor site) was determined by means of a bioassay in animals on the above regimes.

4. Folic acid deficiency is associated with marked decrease in the growth of Rous sarcoma.

5. This is characterized by a marked increase in the size of the tumor cell (nucleus and cytoplasm), hydropic swelling of the nucleus, and apparent concentration of the chromatin.

6. These features are also seen in the immature cells of the bone marrow and the gland cells of the intestine of the deficient animals.

7. Rous sarcoma contains considerably more folic acid than the tissues of the tumor site. This relationship is maintained even in the presence of folic acid deficiency or folic acid excess in the diet.

REFERENCES

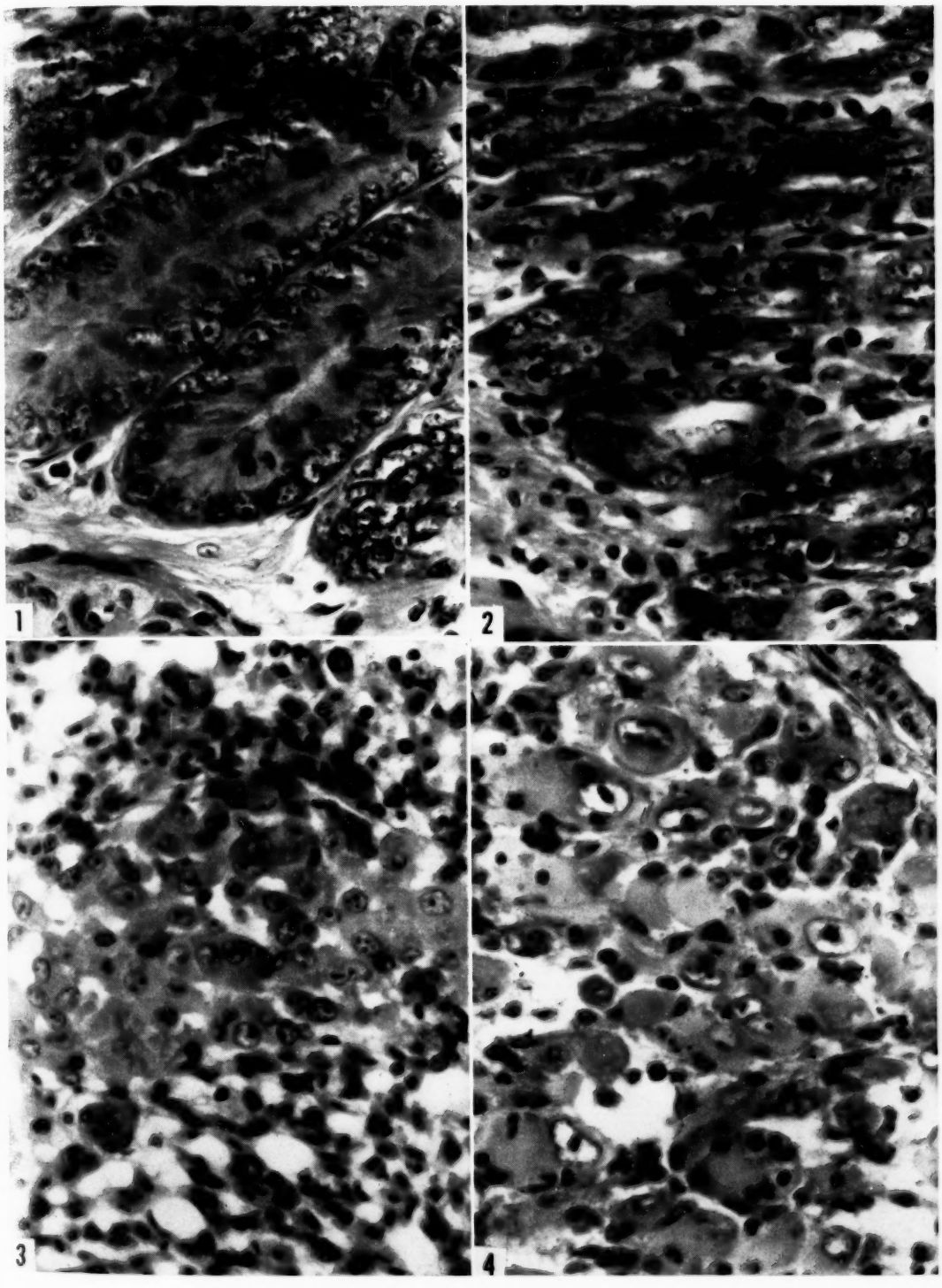
1. LEVINE, M. The Cytology of the Tumor Cell in Rous Chicken Sarcoma. Am. J. Cancer, **36**:276–302, 386–430, 581–602; **37**:69–107, 1939.
2. POLLACK, M. A.; TAYLOR, A.; and WILLIAMS, R. J. B Vitamins in Human, Rat and Mouse Neoplasms. Studies on the Vitamin Content of Tissues. II, pp. 56–71. Austin: The University of Texas Publication, 1942.
3. ROUS, P., and MURPHY, J. B. The Histological Signs of Resistance to a Transmissible Sarcoma of the Fowl. J. Exper. Med., **15**:270–86, 1912.
4. TEPLY, L. J., and ELVEHJEM, C. A. The Titrimetric Determination of "*Lactobacillus casei* Factor" and "Folic Acid." J. Biol. Chem., **157**:303–9, 1945.
5. WOLL, E. The Rous Chicken Sarcoma in Birds Treated with Folic Acid and Its Derivatives: A Pathological Study. Trans. N.Y. Acad. Sc., **10**:83–91, 1948.
6. ———. Effects of Folic Acid Deficiency and a Folic Acid Antagonist on Chicks. Arch. Path., **46**:559–66, 1948.
7. WOLL, E., and OLESON, J. J. The Effects of a Folic Acid Antagonist (Aminopterin) on Albino Rats—A Study in the Pathogenesis of Sprue. Brit. J. Exper. Path. (in press).

FIG. 1.—Normal chick duodenum. Hematoxylin and eosin. ×450.

FIG. 2.—Chick duodenum after 7 days on a folic acid-free diet. Note the swollen cells, loss of chromatin detail, and disrupted configuration. Hematoxylin and eosin. ×450.

FIG. 3.—Rous sarcoma in a control bird (15 days). Hematoxylin and eosin. ×450.

FIG. 4.—Rous sarcoma in a bird after 15 days on a folic acid-free diet. Note the marked swelling of the tumor cells, loss of chromatin detail, and hydropic swelling of the nucleus. Hematoxylin and eosin. ×450.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Tracer Studies on the Metabolism of the Gardner Lymphosarcoma

IV. The Conversion of Lactate-2-C^{14} to Alanine, Glutamate, and Aspartate by Tumor and Spleen Cells*

SAUL KIT† AND DAVID M. GREENBERG

(*Division of Biochemistry, School of Medicine, University of California, Berkeley, Calif.*)

INTRODUCTION

Tumor metabolism is characterized by a high rate of lactic acid production under both aerobic and anaerobic conditions. Lactic acid probably represents a metabolic *cul de sac*. Since this substance and its precursor, pyruvic acid, are normally metabolized by way of the Krebs cycle, considerable interest has been focused in recent years upon the reactions of the citric acid cycle in tumor tissues.

Early observations by Potter and Le Page (7) suggested that "oxalacetic acid oxidase" activity might be very low in tumor tissues. Recently, however, Weinhouse *et al.* (11) have shown that C^{14}-labeled fatty acids are oxidized to $C^{14}O_2$ in three mouse tumors and that quinidine citrate-C^{14} could be isolated from the reaction mixture. It was also shown by Weinhouse and co-workers (12) that the condensing enzyme, *cis*-aconitase, and isocitric dehydrogenase were active in those tumors. Kit and Greenberg (4) obtained evidence indicating that the Krebs cycle was operating in the Gardner lymphosarcoma. The most recent work from the Wisconsin laboratories is in agreement with these findings (8).

Radioactive carbon-labeled lactic acid provides an excellent tool with which to study this problem further. Experiments were therefore carried out which were designed to throw additional light upon the pathways by which lactic acid is metabolized in the lymphosarcoma cells and normal mouse spleen cells. Interesting differences between these tissues in the conversions of the labeled carbon of lactic acid to alanine, glutamate, and aspartate were observed and are presented here.

METHODS

Female C3H mice, weighing 22–25 gm., were used. Food was withdrawn 3 hours prior to the time that the mice were sacrificed. The general methods used in the preparation of the cell suspensions from pooled tumors or spleens have been reported previously (3). In this study, cell suspensions were centrifuged for 5 minutes at 2,000 r.p.m. in an International refrigerated centrifuge. The cells were resuspended and twice recentrifuged, and in each case the bottom-most 0.2 ml. of packed cells were discarded. In this way, many of the erythrocytes which contaminated the spleen cell suspensions were eliminated, although it was recognized that the two washes were partially removing soluble components of metabolic significance.

Incubations were conducted in Warburg flasks with 0.2 ml. of 20 per cent KOH in the center wells and 0.2 ml. of 20 per cent trichloroacetic acid in the sidearms. The main compartment contained 0.5 ml. of the cell suspension ($100–150 \times 10^6$ cells) and 0.1 ml. of 0.009 M lactate (84,000 counts/min) or 0.1 ml. of a modified Krebs-Ringer phosphate solution. After 150 minutes at 37.5° C. with air as the gas phase, the incubation was terminated by tipping in acid from the sidearm. The further procedures in the washing of the tissues and the

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† Predoctoral Research Fellow, National Cancer Institute, National Institutes of Health, Public Health Service, 1950–51. Present address: Chemical Division, Dept. of Medicine, University of Chicago, Chicago, Ill.

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preparation of the protein and respiratory CO_2 for radioactive assay have been described (3, 4).

The alcohol, ether-alcohol, and ether washes were pooled, concentrated on a steam bath, transferred to shallow aluminum cups, evaporated to dryness under an infrared lamp, and counted with a flow-gas counter.

The trichloroacetic acid extracts and washes were pooled, extracted 4 times with ether, and then concentrated *in vacuo* to 5–10 ml. The concentrate from each incubation was chromatographed on 120 cm. × 12 mm. columns containing Dowex-50, 250–500 mesh, with the automatic fraction collector described by Peterson, Lien, and Greenberg (6).[1]

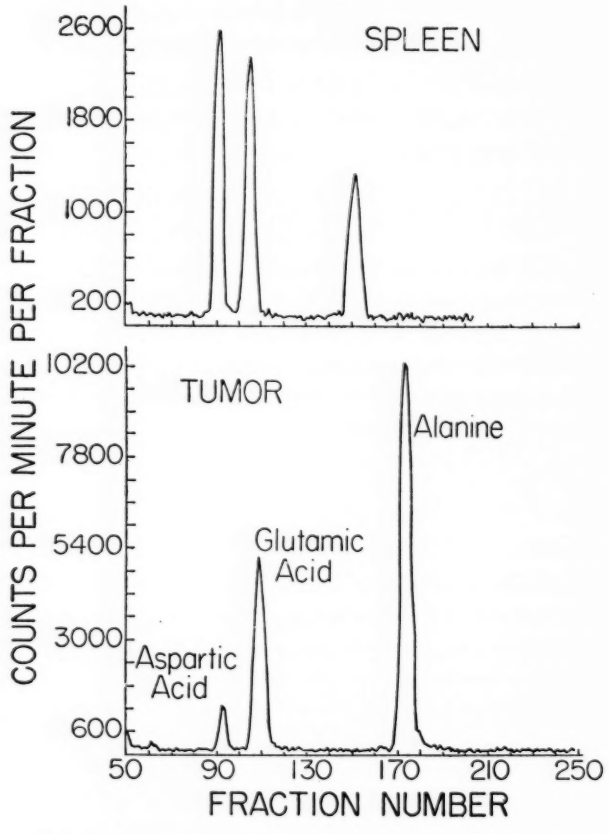

CHART 1.—Fractionation of trichloroacetic acid extracts from incubations with normal spleen and lymphosarcoma cells on Dowex-50; solvent: 1.5 N HCl. Data plotted are of experiment 2 for the tumor and 4 for the spleen (see Table 1).

The fractions (1.5–2 ml.) were collected in polyethylene cups, evaporated to dryness, and the radioactivity determined with a flow-gas counter. Standards were prepared in the polyethylene and aluminum cups in order to obtain values for self-absorption and other corrections.

Lactic acid determinations were performed by the method of Barker and Summerson (1).

The lactate-2-C^{14} used in these experiments was obtained from Dr. Bert Tolbert (University of California Radiation Laboratory) as the zinc salt and was freed from this cation by passage through a short column containing a cation exchange resin.

RESULTS

Chromatograms.—The peaks of radioactivity obtained after chromatographing with Dowex-50 are illustrated in Chart 1. The first 40–50 fractions contained a broad, high peak of radioactivity, caused by the unreacted lactic acid or by other acidic substances such as pyruvic and acetic acids. These cups were counted in only one experiment. Three amino acids were further verified by paper chromatography (Whatman No. 1) with butanol-water-acetic acid (100:50:22.5) and phenol-water (50 gm.:19 ml.) as the solvents. However, paper chromatograms from the aspartic and glutamic acid cups, run with the second solvent, each showed an additional weak spot containing radioactivity. These two spots were close to the origin and may have been due to acidic peptides.

The total counts in each of the peaks are shown in Table 1. It may be noted that the pattern of radioactivity is strikingly different in the tumor incubations than in those of the spleen cells. In the tumor, radioactivity due to alanine was double that due to glutamic acid, while that due to aspartate was comparatively low. In the spleen cell incubations the radioactivity due to the aspartate was almost as great as that due to glutamate, while the alanine peak showed the least radioactivity. The tumor cells formed more alanine-C^{14} and glutamate-C^{14} from lactate-C^{14} than did the spleen cells.

Endogenous lactic acid.—It was of interest to determine the extent to which endogenous lactic acid was diluting the radioactive lactic acid added to each flask. Analyses (4) showed that at the start of an incubation, the spleen cells contained approximately 16 μg. of lactic acid, while the tumor cells contained about 60 μg. Since 80 μg. of lactate-C^{14} was added to each flask, it was clear that this radioactivity was not significantly diluted by lactic acid from the spleen cells but that the dilution due to the lactic acid of the tumor cells was a factor of importance. It is also to be emphasized that, although the incubations were conducted aerobically, the glycolytic formation of lactic acid by the tumor probably exceeded the formation of lactate by the spleen cells.

Respiration.—In order to throw further light on the results, simultaneous observations were made on the oxygen consumption, $C^{14}O_2$ pro-

[1] The generous assistance of Mr. Oliver G. Lien, Jr., in the chromatographic methods is gratefully acknowledged.

duction, and radioactivity of protein and lipid in the cells. In the four experiments in which oxygen uptake was measured, the average uptake per experimental flask was 137 and 170 μl. for the spleen cells and 159 and 186 μl. for the tumor cells. The presence of lactic acid raised the oxygen consumption of the spleen cells 20 per cent but did not alter the respiration of the tumor cells. This may perhaps be attributed to the fact that the tumor cells already contained considerable lactic acid, as indicated above.

Radioactivity in lipids and protein.—Lactic acid may be converted to lipids at an appreciable rate in liver tissue. Under the conditions of these experiments, however, the total radioactivity due to lipids was negligible in both the lymphosarcoma and spleen cells. It would therefore appear that this represents a minor pathway for disposing of the lactic acid by these cells.

The radioactivity to be found in the protein is a function of the rate at which lactate is converted to amino acids. It was found that the labeling of the proteins increased throughout the incubation. The specific activity of the protein from the tumor cells was somewhat lower than that of the spleen cells. However, if one corrected for the dilution caused by the endogenous lactic acid produced by the tumor cell, one found that the incorporation into the proteins of the two cell types was approximately equal. About equal rates of incorporation of radioactive alanine and glycine into the proteins of these cells were reported in a previous publication (5).

Respiratory $C^{14}O_2$.—In the experiments with the spleen cells, 15 and 18 per cent of the added radioactivity was found in the respiratory CO_2. The figures for the lymphosarcoma were 10 and 19 per cent. Thus, both the normal spleen cells and the tumor cells oxidized lactate-2-C^{14} to an appreciable extent.

DISCUSSION

Experiments already reported (4) indicated that acetate-1-C^{14} was oxidized to $C^{14}O_2$ by the cells of the spleen and Gardner lymphosarcoma. The O_2 utilization of the tumor cells was stimulated by succinic acid, and leucine-2-C^{14} was also shown to be partially oxidized to $C^{14}O_2$. The demonstration that lactate-2-C^{14} is oxidized to $C^{14}O_2$ and that part of the radioactivity may be found in alanine, aspartic, and glutamic acids provides further evidence that the oxidations of the Krebs cycle proceed to an appreciable extent in both the spleen and lymphosarcoma cells.

Alanine contained less radioactivity than the dicarboxylic amino acids in the incubations with the spleen cells. This, coupled with the fact that about the same amount of radioactivity was found in the latter two amino acids, suggests that the enzymes which metabolized lactic acid to CO_2 were "harmoniously geared" in the spleen. This is further supported by our earlier experiments (4), since it was found that when spleen cells were incubated with oxalacetic and acetic acids, no citrate accumulated unless fluoroacetate was added. Thus, citric acid was being metabolized as rapidly as it was formed. On the other hand, when the tumor cells were incubated with acetic and oxalacetic acids, citrate accumulated in the absence of fluoroacetate, and the addition of fluoroacetate induced only a small increase in citric acid. Thus, the tumor was apparently capable of forming citric acid more rapidly than it was able to metabolize it.

TABLE 1

DISTRIBUTION OF THE RADIOACTIVITY IN ALANINE, ASPARTIC, AND GLUTAMIC ACIDS FOLLOWING INCUBATION WITH LACTATE-2-C^{14}

Exp.	Tissue	Glutamate counts/min*	Per cent of added counts	Aspartate counts/min*	Alanine counts/min*	Ratio Glut.:Asp.:Alan.
1	Tumor	20,900	4.9	3,660	35,900	1:0.18:1.72
2	Tumor	24,500	4.2	4,440	55,100	1:0.18:2.25
3	Spleen	13,750	2.0	10,430	9,480	1:0.76:0.69
4	Spleen	13,070	2.6	11,100	7,660	1:0.85:0.59
5†	Spleen	7,440	1.0	7,180	4,640	1:0.97:0.62

* Counts per minute. The magnitude of the counts with the flow-gas counter is about 3 times as great as with the mica end-window tube reported in the previous papers of this series.

† Incubated at 30° C.

Table 1 shows that 5 times as much radioactivity was found in the glutamate of the tumor as in the aspartate. An accumulation of both citrate and glutamate would be expected if the oxidation of α-ketoglutarate were the rate-limiting step in the Krebs cycle oxidations of the tumor cell. Additional data on the quantity of α-ketoglutaric acid formed and the α-ketoglutaric acid oxidase activity are required to ascertain whether this is correct. It is to be noted that the observed pattern of radioactivity in the lymphosarcoma amino acids would also be found as a consequence of several other factors: (*a*) a high glutamate content

which would serve as a trap when radioglutamate entered the glutamate pool, (*b*) a high level of glutamate-pyruvate transaminase activity in the tumor, (*c*) a low level of malic dehydrogenase in the tumor.

Unfortunately, only limited data are as yet available concerning these points. Semiquantitative determinations of the free amino acid content of mouse lymph nodes and lymphosarcoma tissue were reported by Roberts and Frankel (10). The paper chromatograms prepared by these investigators indicated that the lymphosarcoma cells had much less aspartate, about the same amount of glutamate, and higher levels of alanine than the lymph nodes. As for transaminase activity, the reaction between alanine and α-ketoglutarate is apparently quite slow in lymph nodes and rat or guinea pig spleen (2). The reaction between glutamate and oxalacetate is slowest in the spleen, among eight rat tissues (2). However, Redfield and Barron (9) have reported activity for the rabbit appendix which compares favorably with that of the rabbit kidney.

SUMMARY

When mouse lymphosarcoma or spleen cells were incubated with lactate-2-C^{14}, significant radioactivity was found in the respiratory CO_2. The only amino acids strongly labeled were alanine, glutamate, and aspartate; and the protein was also significantly labeled. Negligible radioactivity was found in the fats. In the spleen cells, the glutamate and aspartate contained almost equal radioactivity, while the alanine was less radioactive. On the other hand, in the lymphosarcoma cells the alanine had twice as much radioactivity and the aspartate one-fifth as much as the glutamate.

REFERENCES

1. Barker, S. B., and Summerson, W. H. The Colorimetric Determination of Lactic Acid in Biological Material. J. Biol. Chem., **138**:535–54, 1941.
2. Braunstein, A. E. Transamination and the Integrative Functions of the Dicarboxylic Acids in Nitrogen Metabolism. Advances in Protein Chemistry, **3**:1–52, 1947.
3. Farber, E.; Kit, S.; and Greenberg, D. M. Tracer Studies on the Metabolism of the Gardner Lymphosarcoma. I. The Uptake of Radioactive Glycine into Tumor Protein. Cancer Research, **11**:490–94, 1951.
4. Kit, S., and Greenberg, D. M. Tracer Studies on the Metabolism of the Gardner Lymphosarcoma. II. Energy-yielding Reactions and Amino Acid Uptake into Protein of the Tumor Cell. Cancer Research, **11**:495–99, 1951.
5. ———. Tracer Studies on the Metabolism of the Gardner Lymphosarcoma. III. The Rate of Radioactive Glycine and Alanine Uptake into the Protein of Lymphosarcoma Cells and Normal Spleen Cells. Cancer Research, *Ibid.*, pp. 500–04.
6. Peterson, E. A.; Lien, O. G., Jr.; and Greenberg, D. M. A Simple Fraction Collector for Column Chromatography. Anal. Chem. (in press).
7. Potter, V. R., and Le Page, G. A. Metabolism of Oxalacetate in Glycolyzing Tumor Homogenates. J. Biol. Chem., **177**:237–45, 1949.
8. Potter, V. R., and Lyle, G. G. Oxidative Phosphorylation in Homogenates of Normal and Tumor Tissues. Cancer Research, **11**:355–60, 1951.
9. Redfield, R. R., and Barron, E. S. G. The Metabolism of the Appendix (Rabbit). Arch. Biochem., **26**:275–86, 1950.
10. Roberts, E., and Frankel, S. Free Amino Acids in Normal and Neoplastic Tissues of Mice as Studied by Paper Chromatography. Cancer Research, **9**:645–48, 1949.
11. Weinhouse, S.; Millington, R. H.; and Wenner, C. E. Occurrence of the Citric Acid Cycle in Tumors. J. Am. Chem. Soc., **72**:4332–33, 1950.
12. Wenner, C. E.; Spirtes, M. A.; and Weinhouse, S. Enzymes of the Citric Acid Cycle in Tumors. J. Am. Chem. Soc., **72**:4333, 1950.

Effects of Low Temperatures on Mammary Carcinomas with and without the Mammary Tumor Milk Agent

L. W. Law

With the technical assistance of Mr. J. H. Miller

(*National Cancer Institute, Bethesda, Md.*)

Recently, Gye and co-workers (12, 13, 17, 18) have published a series of reports indicating that sarcomas and carcinomas of mice, when frozen at −79° C. or when frozen and dried, retain the ability to induce, *de novo*, sarcomas in the connective tissue or carcinomas in the mammary tissues of the host—depending upon the tumor used.

The observations of Mann (17, 18) are particularly pertinent to the present report. She subjected a series of spontaneous mammary carcinomas and a transplantable carcinoma of hybrid mice to a temperature of −79° C. for varying periods of time and studied their fate when transplanted into mice of the strain of origin of the tumor under investigation. More than half the animals inoculated with frozen tumor mince developed tumors; these appeared in both males and females inoculated in the mammary gland region, but failed to appear following intraperitoneal inoculations. The following conclusions were drawn from this work: (*a*) Under normal conditions the mammary tumor milk agent is present in a latent form in the body of the mouse and is normally activated only after long continued action of estrin. Freezing of mammary carcinoma tissue at −79° C., however, liberates the virus in its active form. (*b*) In the active form, the virus is shown to be "selectively infective," inducing new tumors in mice of either sex when inoculated into mammary tissue but remaining inactive when inoculated into the peritoneal cavity. (*c*) Evidence that the tumors produced were not transplants but true infections was reported to be demonstrated by the fact that the longer the mammary tumor tissue was subjected to low temperature, the greater was the percentage of tumors. Mann also reported that mammary carcinomas could be produced by the injection of tumor material that had been frozen and dried (18).

The interpretation given by Gye on the numerous observations reported is of interest, since the results of ultrafiltration experiments with mammalian tumors, except mammary carcinoma in mice, have been consistently negative.

The interpretation given by Mann of her results is of interest, since they are at variance with results published by workers interested in the biologic behavior of the mammary tumor milk agent, especially the work of Bittner (17) and Andervont (1). It has been shown that the milk agent has the properties of a virus and remains active following filtration, lyophilization, desiccation and storage for long periods of time. When administered in cell-free extracts by numerous routes, mammary tumors develop in appropriate test mice in many different mammary gland sites and only after a latent period of many months. Mammary tumors do not necessarily develop at the site of parenteral administration, and tumors are not hastened in appearance when cell-free extracts are given subcutaneously as compared with administration intraperitoneally.

Several investigators have questioned the validity of the prevalent interpretations (2–4) of these experiments. Hirschberg and Rusch (15) have commented on the interpretations in view of the numerous reports in the literature indicating survival of normal tissues and the growth of tumor tissue in tissue culture after freezing. Walsh *et al.* (23) have shown, using Sarcoma 37, that freezing at temperatures as low as −190° C. does not appreciably alter the latent period nor the morphologic characteristics, and they have demonstrated, significantly, apparent cell survival following freezing.

A more positive approach to the question of virus etiology, especially in mouse sarcomas and carcinomas, has been made by investigators at the University of Leeds (10, 19, 20) and in mouse carcinoma by Bittner and Imagawa (8). It would

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appear that the most important contribution by the Leeds investigators was the report that mouse sarcoma cells that were frozen and desiccated showed active growth *in vitro* after reconstitution in glucose (20). Bittner and Imagawa (8), using a transplantable mammary tumor of hybrid origin and one which arose in C3H mice, found that tumor tissue which had been frozen to −79° C. produced mammary tumors only in mice that were genetically susceptible to fresh tissue grafts of the tumor tested. Furthermore, it was shown that cell-free extracts of the frozen mammary tumor mince failed to induce tumors within the expected interval and that cell-free extracts of the frozen tissues were no more active than extracts of the fresh tissue of the same tumor.

The studies to be reported here are concerned with (*a*) the behavior in transplantation of a typical mouse mammary adenocarcinoma known not to contain the mammary tumor milk agent, compared, according to the procedures outlined by Mann (17), to the behavior of a typical mammary carcinoma known to contain the milk agent; and (*b*) the behavior in transplantation, following freezing, of these two mammary tumors (with and without the milk agent) in mice of different genetic constitutions. According to the interpretations given by Mann (17), one would not expect induction of tumors in appropriate test mice following the freezing of tumor material not containing the "latent" mammary tumor milk agent, since her thesis is based upon transformation of a "latent" to an "active" virus following freezing. Secondly, upon introduction of frozen tissue mince which, it is assumed, contains active virus, the tumors which appear should be induced in the mammary tissues of the host. That is, the tumor should be of the same genetic constitution as the host. An attempt is made here to identify the genetic constitution of the tumors arising in mice of different genetic constitutions in order to determine whether they (*a*) result from transfer of living cells or (*b*) are actually induced by "active" virus.

MATERIALS AND METHODS

The mammary tumor designated MT-8[1] arose in a 16-month-old female mouse of the milk agent-free C3Hb line. Originally, this tumor showed large areas of reproduction of small mammary acini with evidence of solid structure in many areas. There was a high degree of keratinization at this time (Fig. 1). Following continuous passage in C3Hb mice the squamous-cell elements largely disappeared, and at the time this tumor (the thirty-fifth transfer generation) was used, it was typically of the adenocarcinomatous type (Figs. 2, 3). The origin and characteristics of the particular C3Hb mice in which this tumor arose have been described by Heston *et al.* (14). Appropriate biologic assays for the mammary tumor agent in numerous mammary tumors of this line have been consistently negative (14). Biologic tests for the presence of the milk agent were made at the second transfer generation of this tumor. Tumor tissue was minced, homogenized, and diluted 1:5 by weight with saline. Following centrifugation for 10 minutes at 3,000 r.p.m., the supernatant was filtered through a tested Berkefeld filter and injected (0.2 cc.) into B alb C (C) strain female mice 2–3 weeks of age. The eleven test animals have remained tumor-free for 19 months. This tumor has been carried exclusively in agent-free C3Hb mice. It has grown progressively in all mice inoculated and kills in approximately 30 days.

Mammary tumor L 1221 arose in a C3H breeding female, of the F_{15} generation,[2] when 7 months of age. This mammary tumor was an adenocarcinoma, the common type found in C3H mice. Large solid nests of cells were commonly found with evidence in some areas of reproduction of the acinar structure. It has been carried since its origin by serial transfer in C3Hb mice—that is, in mice free of the mammary tumor milk agent. It has grown progressively in all mice inoculated, killing in approximately 21 days. Biologic tests for the presence of the milk agent were made at the fifth, tenth, and twentieth transfer generations. A 20 per cent Berkefeld filtrate in saline was injected, 0.2 cc. intraperitoneally, into 2–3-week-old B alb C (C) female mice. Seventeen mammary tumors appeared in 31 test mice at an average age of 9.2 months, with approximately equal distribution of tumors at each transfer tested. Tissue from the 38th transfer generation was used in this experiment.

Mammary tumors MT-9 and E 5640 were both found to be inactive following the freezing procedure used here. Both tumors arose in milk agent-free mice of the C3Hb line, and tumor filtrates from each failed to induce tumors in appropriate test mice. Tumor MT-9 closely resembled in structure tumor L 1221, an adenocarcinoma with large solid nests of cells. Tumor E 5640 contained many squamous-cell elements in the

[1] The three mammary tumors which arose in mice of lines C3Hb, MT-8, MT-9, and E 5640 were obtained from Dr. W. E. Heston of this Institute. The first two tumors were from line 3 and E 5640 was from line 1, C3Hb mice.

[2] C3H strain mice were obtained from Dr. W. E. Heston, who, in turn, obtained the strain from Dr. H. B. Andervont. F_{15} indicates the number of brother × sister generations of inbreeding since the mice were obtained from Dr. Andervont. All C3H and C3Hb mice used in this study were derived from this source.

original sections, but these disappeared upon continuous passage in line C3Hb mice.

Test animals used for transplantation were C3Hb, milk agent-free mice, either those described by Heston (14) or our own subline, in which a mammary tumor incidence of 11.1 per cent in breeding females (average age: 15.6 months) has been found. The F_1 hybrids were obtained by crossing RIL and C3Hb strains of mice. RIL mice were also used for testing at various transfer generations. All mice receiving frozen tumor mince were from 4 to 6 weeks of age.

Tumor tissues were prepared according to the method of Mann (17). Tissue was removed aseptically, freed of any necrotic material, minced, and passed through a specially prepared Craigie mincer (9).[3] Mincing of tissue was accomplished by using first the coarse plunger and then the fine plunger. The mince was then diluted with an equal volume of 5.3 per cent glucose, sealed in glass ampules, 3 cc. per vial, and then cooled slowly in a mixture of cellosolve and dry ice, the dry ice being added slowly so that approximately 10 minutes were required to reach −79° C. The vials were stored in a thermos jug which was in turn placed in a deep freeze. Ampules were removed one at a time as needed. As soon as the contents were thawed at room temperature, inoculations were done by use of a size 19 hypodermic needle. The amount given in single subcutaneous and intraperitoneal inoculations was 0.1 cc. per mouse. When two inoculations were given simultaneously, in the right and left axillary regions, 0.05 cc. in each region or 0.1 cc. per mouse was given. This represents 0.05 cc. of tumor mince per mouse.

[3] The pressure mincer was machined by Mr. Laurence Crisp, of the National Institutes of Health Instrument Shop, out of stainless steel, according to the design (7) and personal instructions from Craigie. In all preparations of tumor material the coarse plunger was used first followed by the fine plunger. The longitudinal grooves on the coarse plunger were 0.5 mm. deep and 0.5 mm. wide; and the grooves on the fine plunger were 0.0625 mm. deep and 0.0625 mm. wide. There were thirty grooves in the coarse plunger and forty grooves in the fine plunger, triangular in cross section, and cut at an angle of 60°.

EXPERIMENTAL RESULTS

It can be seen (Table 1) that two mammary tumors, MT-9 and E 5640, were completely susceptible to freezing. In preliminary tests, tumor L 1221, containing the milk agent, was also found to be inactive in transplantation following freezing at −79° C. for 24 hours, if the tissue mince was frozen quickly by immersion directly into the dry ice–cellosolve mixture. Passey and Dmochowski (19) observed that mammary tumors of mice apparently containing the milk agent, especially those carried in transplant, were particularly susceptible to freezing. Thus, it would appear that there is no direct relationship between absence of the mammary tumor milk agent and susceptibility to freezing.

TABLE 1

BEHAVIOR IN TRANSPLANT FOLLOWING FREEZING AT −79° C. OF SEVERAL MAMMARY TUMORS

Tumor designation	Transfer used	Strain of mouse	Milk agent	Diluent	Time frozen (hrs.)	Route of inoculation	Mice Sex	Mice No.	No. of tumors*	Death from tumor (days)
L 1221	G-38	C3H	Present	5.3 per cent glucose	1, 24	Subcut.	M and F	27	14	67.6 (41–96)
						Intrap.	M and F	8	0	
MT-8	G-35	C3Hb	Absent	5.3 per cent glucose	1, 24, 72	Subcut.	M and F	33	29	70.9 (50–131)
						Intrap.	M and F	14	13	64.2 (47–75)
MT-9	G-20	C3Hb	Absent	5.3 per cent glucose	24, 96	Subcut. and Intrap.	M and F	28	0	
E 5640	G-48	C3Hb	Absent	5.3 per cent glucose	1, 24, 96	Subcut. and Intrap.	M and F	51	0	

*Indicates, throughout tables, progressively growing transplants causing death of host animals.

Forty-three out of 47 C3Hb and RIL/C3Hb F_1 mice of both sexes developed progressively growing tumors following inoculation of tumor MT-8, without the milk agent, frozen at −79° C. for 1–72 hours (Table 2). Subcutaneous masses developed at the site of transplantation (Fig. 2). Among four cases inoculated into both right and left axillary regions, all developed tumors on each side at the site of transplantation. Among the fourteen animals receiving intraperitoneal inoculations, all of which resulted in progressively growing neoplasms, tumor masses were observed free in the cavity with no evidence that these were connected to the peritoneum at the site of inoculation (Fig. 3).

Following progressive growth of frozen MT-8 tumor in RIL/C3Hb F_1 hybrids, the tumor tissue

TABLE 2

DEVELOPMENT OF MAMMARY TUMORS IN TEST MICE FOLLOWING INOCULATION OF FROZEN TUMOR TISSUE OF C3Hb TUMOR, MT-8 (WITHOUT THE MAMMARY TUMOR MILK AGENT)

Time frozen at −79° C. (hrs.)	Age inoc. (wks.)	Mice inoculated: Route	Sex	Strain	No.	No. tumors	Days at death
1	4–6	Subcut.	♂	C3Hb	6	6	80.5
	"	"	♀	RIL/C3Hb F_1	3	3	56.3
	"	Intrap.	♀	C3Hb	5	5	60.2
24	4–6	Subcut.	♂	C3Hb	5	5*	79.8
	"	"	♀	C3Hb	5	4*	73.0
	"	"	♀	RIL/C3Hb F_1	3	3†	55.0
	"	Intrap.	♀	C3Hb	5	5	64.5
72	4–6	Subcut.	♀	C3Hb	4	2	57.0
	"	"	♂	C3Hb	4	4	71.2
	"	"	♀	RIL/C3Hb F_1	3	2†	82.0
	"	Intrap.	♀	C3Hb	4	4	69.0

* Two animals in each group inoculated on both right and left sides. Progressively growing growths appeared at each site of transplant.

† Carried serially in transplant. See Table 4.

TABLE 3

DEVELOPMENT OF MAMMARY TUMORS IN TEST MICE FOLLOWING INOCULATION OF FROZEN TISSUE OF C3H TUMOR L 1221 (WITH THE MAMMARY TUMOR MILK AGENT)

Time frozen at −79° C. (hrs.)	Age inoc. (wks.)	Mice inoculated: Route	Sex	Strain	No.	No. tumors	Days at death with tumor
1	4–6	Subcut.	♂	C3Hb	5	1	67.0
	"	"	♀	C3Hb	5	1	84.0
	"	"	♂	RIL/C3Hb F_1	3	1	84.0
	"	Intrap.	♀	C3Hb	4	0	
24	4–6	Subcut.	♂	C3Hb	7	3	62.0
	"	"	♀	C3Hb	4	1	71.0
	"	"	♂	RIL/C3Hb F_1	3	3*	61.7
	"	Intrap.	♂	C3Hb	4	0	

* Carried serially in transplant. See Table 4.

was then transferred in five consecutive passages through RIL/C3Hb F_1 hybrid hosts. At the first and last passages, inoculation into RIL and C3Hb strains and into RIL/C3Hb F_1 hybrids was accomplished in order to determine the genetic constitution of the tumor tissue. It can be seen (Table 4) that progressively growing tumors were obtained in both C3Hb mice, the strain of origin of the tumor, and in hybrid mice, with tissue obtained from the 24-hour and 72-hour series, respectively; and no growths were obtained in RIL mice.

The behavior at transplants of tumor L 1221, containing the mammary tumor milk agent, following freezing at −79° C. for periods of 1 hour and 24 hours, is shown in Table 3. Ten progressively growing tumors were obtained in the 35 mice used in this experiment. The results obtained on all mice which had received intraperitoneal inoculations were negative for 120 days. The mean age at death from tumor was considerably longer than that observed for mice bearing untreated tumor material.

Tumor tissue which grew progressively in a hybrid C3Hb/RIL F_1 male, inoculated with frozen tumor mince in the 24-hour series, was transplanted serially for five generations in C3Hb/RIL F_1 hybrids. It can be seen from Table 4 that tumors were produced which caused the death of all C3Hb and C3Hb/RIL F_1 hybrid mice, but the results in RIL mice were negative.

In contrast to the results of Walsh *et al.* (23), which indicated a loss in growth potential of frozen Sarcoma 37 after subsequent passage, both tumors MT-8 and L 1221 showed, in fact, an increase in growth potential, as measured by mean survival time, in subsequent passages after freezing (Table 4).

TABLE 4

FATE IN TRANSPLANTATION OF PREVIOUSLY FROZEN MAMMARY TUMORS MT-8 (WITHOUT MILK AGENT) AND L 1221 (WITH MILK AGENT) TUMORS CARRIED SERIALLY IN RIL/C3Hb F_1 MICE

Mammary tumor	Expt.	Transfer Generations: 1 Strain inoc.	1 No. mice*	1 No. tumors	1 Days death	2–4 Strain inoc.	2–4 No. mice*	2–4 No. tumors	2–4 Days death	5 Strain inoc.	5 No. mice*	5 No. tumors	5 Days death
MT-8	I (24-hour tissue)	C3Hb	3	3	61					C3Hb	7	7	27.4
		RIL/C3Hb F_1	3	3	50	RIL/C3Hb F_1	9	9	42.0	RIL/C3Hb F_1	7	7	34.7
		RIL	3	0						RIL	7	0	
	II (72-hour tissue)	C3Hb	3	3	52.7					C3Hb	3	3	33.0
		RIL/C3Hb F_1	3	3	55.0	RIL/C3Hb F_1	9	9	44.6	RIL/C3Hb F_1	3	3	39.1
		RIL	3	0						RIL	3	0	
L 1221	I (24-hour tissue)	C3Hb	3	3	53.3					C3Hb	7	7	43.7
		RIL/C3Hb F_1	3	3	32.5	RIL/C3Hb F_1	9	9	51.3	RIL/C3Hb F_1	7	7	37.4
		RIL	3	0						RIL	7	0	

* Mice of both sexes used. No sex differences noted among various groups.

DISCUSSION

Some neoplasms, notably a kidney tumor of the leopard frog, the Shope papilloma, and a series of sarcomas and lymphoid tumors of the fowl, have a virus as their causative agent. Thus, filtrates of such tumors when injected into appropriate hosts produce tumors morphologically similar to the original tumor. Filtrates have been found to be ineffective for most mammalian tumors, but many virus workers believe that only a quantitative difference exists between tumors of the fowl, for example, and tumors of the mammal and that probably technical difficulties (either viruses of low titer or of high lability) prevent successful filtration of the spontaneous or transplantable tumors of mammals. Thus, in the work of Gye and associates, recourse has been taken in other technics, and the burden of evidence rests on histologic observations, these workers contending that living cells are never found in their frozen or lyophilized preparations.

It seems clear, especially as regards the mammary tumors of the mouse, that there are several critical tests available for determining whether Gye and associates were in fact dealing with "active" virus or were transmitting cellular material. We have attempted to apply two of these tests in the present work.

Many influences have been found to be effective in the etiology of mammary tumors in mice. One of these, the mammary tumor milk agent, has many of the characteristics of a virus. When given to appropriate test mice, as a cell-free filtrate, mammary tumors appear after a fairly long latent period, usually at least 6 months. The tumors appear at random among the various mammary glands, they do not necessarily arise at the site of parenteral injection, and administration of the material subcutaneously, thus near the site of the mammary gland, does not accelerate the appearance of tumors in comparison with administration intraperitoneally. It has also been found (14) that mammary tumors will appear, especially among breeding females, in a line of mice, C3Hb, which has been freed of the agent by removing a litter by cesarean section and foster-nursing on a milk agent-free strain, C57 black. Standard biologic tests for the presence of the milk agent in a large series of mammary tumors arising in these strains have been consistently negative.

If Mann (17) is correct in assuming that an "active" milk-agent virus is liberated following freezing and that this virus then induces mammary cancer in a matter of days, it would not be expected then that mammary tumors such as MT-8, free of the virus, would induce tumors in test mice following the technic employed by her. Since frozen tissue of tumor MT-8 induced 34 tumors in 37 mice in either subcutaneous tissues or in the peritoneal cavity, it would seem that the assumption is not correct. This does not necessarily rule out the possibility of induction of mammary tumors by an agent other than the mammary tumor milk agent.

A more critical appraisal of Mann's assumption can be made by studying the behavior in transplants of tumors arising in mice of known genetic constitution following the inoculation of frozen tumor mince. This has been done with the MT-8 tumor, without the milk agent, and with tumor L 1221 containing the agent. It is known from numerous studies (6, 16), using different morphologic types of tumors, that susceptibility to transplantation is determined by multiple dominant genes which have been designated "histocompatibility genes" (21). Certain of these genes determining susceptibility have been located in specific chromosomes of the mouse (11). Thus, it can be shown that tumors which arise in an inbred strain of mouse will grow progressively only in individuals of that strain or in F_1 hybrid animals having as one parent the strain of origin of the neoplasm. Tumors arising in mice of an F_1 generation grow progressively only in mice having that specific F_1 genetic constitution, and growth does not occur in either of the parental strains used in producing the F_1 hybrid (5). The F_1 hybrid received histocompatibility genes (determining tumor susceptibility) from each of the parents, and thus resistance to tumor transplants would be expected in each of the parent strains, since neither would ordinarily possess the necessary histocompatibility genes of the other strain. If an activated virus is responsible for induction of mammary tumors in mammary epithelium, such neoplasms would then be of the same genetic constitution as the host animal. Neoplasms arising in RIL/C3Hb F_1 hybrids, used here, would be F_1 tumors and would grow progressively only in mice of the specific RIL/C3Hb F_1 cross. If living tumor cells are inoculated, following freezing, the tumors which arise should be of the same genetic constitution as the strain of origin—in these experiments, C3H strain (or C3Hb line). Serial transplants of two separate tumors of MT-8 and of one tumor of L 1221, which arose in RIL/C3Hb F_1 hybrids following transfer of frozen tumor mince, have grown progressively when tested in either C3H (C3Hb) mice or in the F_1 hybrids, RIL/C3Hb F_1. This constitutes proof that the tumors in question resulted from the transfer of living cells.

SUMMARY

Four transplantable mammary tumors of the mouse were subjected to freezing at −79° C., following the technic of Mann, for varying periods of time. Two of these failed to elicit progressively growing tumors in test mice.

Mammary tumor L 1221, which contains the milk agent, produced progressively growing tumors in C3Hb and in RIL/C3Hb F_1 hybrid mice following inoculation of tumor mince. No growths appeared following intraperitoneal transfer.

Mammary tumor MT-8, known not to contain the mammary tumor milk agent, also produced progressively growing tumors at the site of inoculation in C3Hb and in RIL/C3Hb F_1 hybrid mice following inoculation of the thawed tumor mince. Tumors were not produced in RIL strain mice. All intraperitoneal inoculations produced massive growths.

Each tumor was tested periodically, following initial growth in an F_1 hybrid, for its ability to grow in mice of C3Hb and RIL strains and in RIL/C3Hb F_1 hybrids. Progressively growing tumors were obtained in both C3Hb and RIL/C3Hb F_1 hybrid mice but not in RIL strain mice.

These results are interpreted to mean that: (*a*) The "active" form of the mammary tumor milk agent is not the causative agent in producing tumors following inoculation of frozen tumor mince.

(*b*) There was found to be no evidence of "selective infectivity" of the milk agent in the experiments reported here.

(*c*) Living tumor cells were actually transferred. The transplantation characteristics for both mammary tumors used in this study were those expected of strain C3H tumors, the strain of origin, and not those expected of tumors induced in F_1 hybrid mice by an "active" agent.

REFERENCES

1. Andervont, H. B. The Milk Influence in the Genesis of Mammary Tumors. A.A.A.S. Research Conference on Cancer, pp. 123–39, 1945.
2. Andrewes, C. H. The Bearing of Recent Work on the Virus Theory of Cancer. Brit. M. J., **1**:81–85, 1950.
3. Anonymous. Transmission of Sarcoma by Dried Tissue (Editorial). Brit. M. J., **1**:531–32, 1949.
4. Anonymous. A Virus in Mouse Cancers? (Editorial). Brit. M. J., **2**:270–71, 1949.
5. Bittner, J. J. A Genetic Study of the Transplantation of Tumors Arising in Hybrid Mice. Am. J. Cancer, **15**:2202–47, 1931.
6. ———. A Review of Genetic Studies on the Transplantation of Tumors. J. Genetics, **31**:471–87, 1935.
7. ———. Inciting Influences in the Etiology of Mammary Cancer in Mice. A.A.A.S. Research Conference on Cancer, pp. 63–96, 1945.
8. Bittner, J. J., and Imagawa, D. T. Assay of Frozen Mouse Mammary Carcinoma for the Mammary Tumor Milk Agent. Cancer Research, **10**:739–50, 1950.
9. Craigie, J. A Pressure Mincer for the Preparation of Tumour Suspension. Brit. J. Cancer, **3**:249–50, 1949.
10. Dmochowski, L., and Millard, A. Cellular Transmission of Mouse Sarcomata with Frozen-dried Tumour Tissues. Brit. M. J., **2**:1136–38, 1950.
11. Gorer, P. A.; Lyman, S.; and Snell, G. D. Studies on the Genetic and Antigenic Basis of Tumor Transplantation. Linkage between a Histocompatibility Gene and 'Fused' in Mice. Proc. Roy. Soc., s. B., **135**:499–505, 1948.
12. Gye, W. E. The Propagation of Mouse Tumours by Means of Dried Tissue. Brit. M. J., **1**:511–15, 1949.
13. Gye, W. E.; Begg, A. M.; Mann, I.; and Craigie, J. The Survival of Activity of Mouse Sarcoma Tissue after Freezing and Drying. Brit. J. Cancer, **3**:259–67, 1949.
14. Heston, W. E.; Deringer, M. K.; Dunn, T. B.; and Levillain, W. Factors in the Development of Spontaneous Mammary Gland Tumors in Agent-free Strain C3Hb Mice. J. Nat. Cancer Inst., **10**:1139–51, 1950.
15. Hirschberg, E., and Rusch, H. P. Comments on Recent Experiments with Frozen and Dried Tissue as Evidence for the Virus Etiology of Tumors. Cancer Research, **10**:335–38, 1950.
16. Little, C. C. The Genetics of Cancer in Mice. Biol. Rev., **22**:315–43, 1947.
17. Mann, I. Effect of Low Temperatures on the Bittner Virus of Mouse Carcinoma. Brit. M. J., **2**:251–53, 1949.
18. ———. Effect of Repeated Freezing and Thawing on Mouse Carcinoma Tissue. *Ibid.*, 253–55, 1949.
19. Passey, R. D., and Dmochowski, L. Freezing and Desiccation of Mouse Tumours. Brit. M. J., **2**:1129–33, 1950.
20. Passey, R. D.; Dmochowski, L.; Lasnitzski, I.; and Millard, A. Cultivation *in Vitro* of Frozen and Desiccated Mouse Tumour Tissues. Brit. M. J., 1134–35, 1950.
21. Snell, G. D. Methods for the Study of Histocompatibility Genes. J. Genetics, **49**:87–108, 1948.
22. ———. Five Alleles at the Histocompatibility-2 Locus in the Mouse as Determined by Tumor Transplantation. Cancer Research, **11**:281, 1951.
23. Walsh, L. B.; Greiff, D.; and Blumenthal, H. T. The Effect of Low Temperature on the Morphology and Transplantability of Sarcoma 37. Cancer Research, **10**:726–36, 1950.

Fig. 1.—Original MT-8 tumor. Adenocarcinoma, with evidence of reproduction of mammary acini. Some stratified squamous epithelium present in original tissue. ×200.

Fig. 2.—First transplant of tumor MT-8 following freezing. Subcutaneous growth in C3Hb mouse. ×200.

Fig. 3.—First transplant of tumor MT-8 following freezing. Intraperitoneal growth in C3Hb mouse. ×200.


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Studies on the Effect of 8-Azaguanine on Sarcoma 37 in Mice*†

MURRAY FINKELSTEIN AND PAUL A. THOMAS

(Department of Pharmacology, the George Washington University School of Medicine, Washington, D.C.)

Since the observations of Kidder *et al.* (8) that 8-azaguanine selectively inhibits the development of three types of mouse neoplasms, many investigators have examined the carcinostatic power of this compound on a variety of cancer tissues in different animal species. It has been found that 8-azaguanine is active against many additional malignant neoplasms (5, 6, 9, 10, 14, 16) but produces little or no effect on others (3, 5, 6, 9, 10, 15, 16). Some investigators (5, 8) have reported a lack of toxicity in the host at doses that inhibit tumor growth; others (6, 16) have not observed a well defined separation of the tumor inhibitory action of 8-azaguanine from systemic effects of the compound as manifested in reduced food intake, weight loss, leukopenia, morphological damage, and death. Few or insignificant histological changes in tumors inhibited by 8-azaguanine have been reported. Kidder *et al.* (8) reported that it did not cause the death of E 0771 cells in C57 mice but only checked their growth. Sugiura and his associates (16), who also examined the effect of 8-azaguanine on E 0771 in C57 strain mice, observed small islands of active tumor tissue surrounded by gelatinous edema and necrosis. The most striking tumor damage was reported by Gellhorn *et al.* (5) and by Shapiro and his associates (14), who examined the histological changes induced in the Brown-Pearce carcinoma in rabbits. They observed that the tumor cells of treated rabbits were larger and fewer in number (5, 14), contained condensations of nuclear material in irregular clumps (5), and that the mitotic index was markedly reduced (14). While Shapiro *et al.* (14) also demonstrated that the mitotic indices of the intestinal epithelia and of the testes of treated rabbits were not affected, indicating a possible selective action of the drug, this inhibition of tumor growth was not completely dissociated from toxicity in the host, inasmuch as there was an appreciable loss in body weight. There were no evident cytological changes in other malignant neoplasms (5).

The present study involves an exploratory examination of the growth and morphological changes of Sarcoma 37 in CAF_1 mice treated with 8-azaguanine and the effects of this compound on the host.

MATERIALS AND METHODS

Aseptic mashes of the rapidly growing Sarcoma 37, prepared according to the method described by Leiter *et al.* (11), were implanted into the right thigh muscles of CAF_1 strain mice, 15–22 gm. in weight. For each experimental group there was a comparable group of untreated control mice bearing implants of the same tumor generation. Tumor volumes were estimated by caliper measurements in three dimensions and are reported here as the average increase in size expressed in cubic centimeters. The effect of the drug on tumor growth was graded as follows: (++), (+), (±) and (−) which corresponds, respectively, to an increase in size of $\frac{1}{4}$ (marked inhibition), from $\frac{1}{4}$ to $\frac{1}{2}$ (moderate inhibition), from $\frac{1}{2}$ to $\frac{3}{4}$ (slight inhibition) and $\frac{3}{4}$ or more (no effect) the increase in size of controls, respectively. Variations in concentrations of injected tumor mash accounted for the variation in size of control tumors between experiments (Chart 1). Within any one experimental series the mash concentration injected was relatively constant. Every mouse was weighed daily, observed for signs of systemic toxicity, examined at autopsy at the termination of an experiment, and each tumor was routinely examined for gross changes. Representative tumors and viscera were fixed in 10 per cent formalin or Zenker-formol solution; sections were stained with hematoxylin and eosin and evaluated histologically.

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The mice received Purina laboratory pellets and water ad libitum.

Tumor age at the time of initiation of prolonged therapy varied from 0 to 5 days; in mice that received a single dose, the tumors were

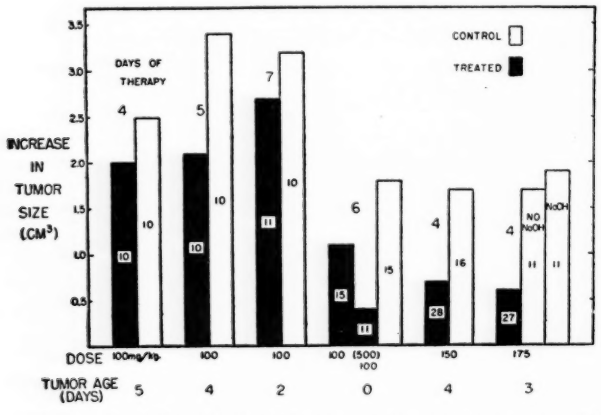


CHART 1.—The effect of 8-azaguanine on tumor growth. Blocked figures within columns indicate number of mice in each group and do not include mice that died during the course of the experiments. (Four mice receiving initial doses of 500 mg/kg died as did three receiving 150 mg/kg/day and one receiving 175 mg/kg/day.) "Tumor Age" refers to age at time of initiation of therapy. The figure in parentheses indicates an initial dose of 500 mg/kg followed by daily doses of 100 mg/kg/day; in this group the initial tumor size was estimated 48 hours after tumor transplant.

5–7 days old. At this time they showed little or no necrosis, except for the original implant. While necrosis occurs spontaneously in Sarcoma 37, this should not be confused with induced necrosis that was observed in mice undergoing 8-azaguanine therapy.

The 8-azaguanine was synthesized, with modifications, according to the method of Roblin *et al.* (13). Analysis by counter-current distribution indicated more than 95 per cent purity, and the ultraviolet absorption spectrum was identical with that described by Cavalieri *et al.* (4). Stock solutions were made in 0.25 N (or less) NaOH, prepared freshly at least once each week. Required dilutions of the drug were made in distilled water, so that the dose for injection was contained in 0.02 cc. per gram of body weight (volume equivalent to 2 per cent total body weight); this dose was administered subcutaneously into the axilla contralateral to the tumor implant.

In several experiments, control mice were divided into two groups. One group received NaOH of the proper dilution subcutaneously, while the other received none. There was no essential difference between these groups, and the results were, therefore, combined. Similarly, sex did not influence the effect of the drug on the tumor or the host; consequently, the results obtained with these two groups were also combined.

RESULTS

Effect on tumor growth.—The average results from 195 mice undergoing 8-azaguanine therapy at different dose levels in which drug administration was initiated at different time intervals after tumor transplant are summarized in Chart 1 and Table 1. It can be seen that at certain dose levels of 8-azaguanine there was relatively significant tumor inhibition. The tumor, however, was not destroyed or completely inhibited, inasmuch as there was progressive but very slow growth. Moreover, following termination of treatment with a dose of 175 mg/kg/day, tumors in mice that were not sacrificed grew rapidly and approached the size of controls. This observation is in accord with the findings of other investigators (5, 8, 16), who demonstrated growth inhibition of different malignant neoplasms.

At a dose of 100 mg/kg/day, the effect was no greater than "slight inhibition" regardless of whether therapy was initiated on the day of tumor transplant or up to 5 days later. Nevertheless, this effect in experiments 2 and 4 was statistically

TABLE 1

EFFECT OF 8-AZAGUANINE ON BODY WEIGHT, SPLEEN WEIGHT AND ON GROWTH OF SARCOMA 37 IN CAF_1 MICE

Exp. no.	Dosage daily (mg/kg)	Total dosage (mg/kg)	Average change body weight (gm.)	Spleen weight (per cent body weight)	Tumor inhibition*
1	100	400	+0.1		−
	0	0	+0.2		
2	100	500	−0.1	0.54	±
	0	0	0.0	0.98	
3	100	700	−2.3	0.75	−
	0	0	−0.8	1.17	
4	100	600	−2.6	0.83	±
	500+100†	1,000	−3.8	0.55	++
	0	0	−1.1	1.50	
5	150	600	−2.4	0.60	+
	0	0	−1.7	1.17	
6	175	700	−3.0	0.69	+
	0‡	0	−0.2	1.40	
	0§	0	−1.8	1.41	

* The grading of tumor inhibition is described in the text.
† Initial dose of 500 mg/kg followed by daily doses of 100 mg/kg.
‡ Controls without NaOH.
§ Controls received injections of NaOH.

significant (probability <0.01). Doses of 150 and 175 mg/kg/day produced statistically highly significant "moderate inhibition." It is interesting to note that the total doses (600–700 mg/kg) administered at this dose level (150 and 175 mg/kg/

day) were similar to the total doses (400–700 mg/kg) administered at the lower level (100 mg/kg/day) but that the former was more effective. Furthermore, even though the lower level therapy was initiated on the day of tumor transplant, as compared to the later initiation of therapy at the higher dose level, the effect was only relatively slight.

Effect on host.—Mortality due to drug toxicity was comparatively low, as indicated in Chart 1.

Table 1 indicates that there was a loss in body weight, during the period of therapy, in both control and treated mice. This observation was closely associated with anorexia that developed in mice that received daily subcutaneous injections of NaOH. There was essentially no change in weight in untreated control animals (Table 1, exp. 6).

Anorexia and loss in body weight were apparently due in part to NaOH injections. This observation was made in both normal and tumor-bearing mice, as is demonstrated in Chart 2. During the 24-hour period following a single subcutaneous injection of 500 mg/kg of 8-azaguanine in NaOH, or NaOH alone, there was marked anorexia in both normal and tumor-bearing mice. Intraperitoneal injections of a similar dose of the drug in water caused but slight anorexia. Essentially complete recovery was evident within 48 hours; however, the weight loss (up to 19 per cent) of both normal and tumor-bearing mice that received 8-azaguanine in NaOH was comparable to mice deprived of food for 48 hours and water for 24 hours. On the second post-injection day, mice that received the drug intraperitoneally in water showed a lesser degree of weight loss (approximately 10 per cent), comparable to the NaOH-treated controls. On the third or fourth post-injection day, the body weight of both drug and NaOH-treated normal and tumor-bearing mice and of those deprived of food began to rise steadily toward that of the untreated controls. The slight loss in weight observed in some untreated controls may possibly have been due to environmental conditions in the laboratory.

There were no signs of diarrhea or other toxic manifestations in this group of mice or in those on a protracted course of 8-azaguanine treatment.

In both the treated and control mice there was a gelatinous edema which was limited to the site of injection, and after multiple injections there often developed an ulceration. These effects, which were apparently due to the relatively high concentration of NaOH, regressed when therapy was terminated. Moreover, there was no apparent correlation between these effects and tumor damage caused by the drug.

The most striking gross effect of repeated doses of the drug on the host was a diminution in the size of the spleen of both normal and tumor-bearing mice, which amounted to approximately 50 per cent of the size of control spleens (Table 1). This decrease in spleen size was seldom seen after a single dose of 500 mg/kg. In addition, there was a pronounced discoloration of the spleen on repeated drug administration, particularly after the higher doses. Whereas the control spleens exhibited the usual healthy red color, the discolored spleens of treated mice were brown and often translucent in appearance. Many of the adrenal glands (18 per cent) of mice that received 175 mg/kg daily were similarly brown in color and often translucent, in contrast to the opaque light color of control adrenal glands. This was not observed in mice receiving daily injections of the lower doses.

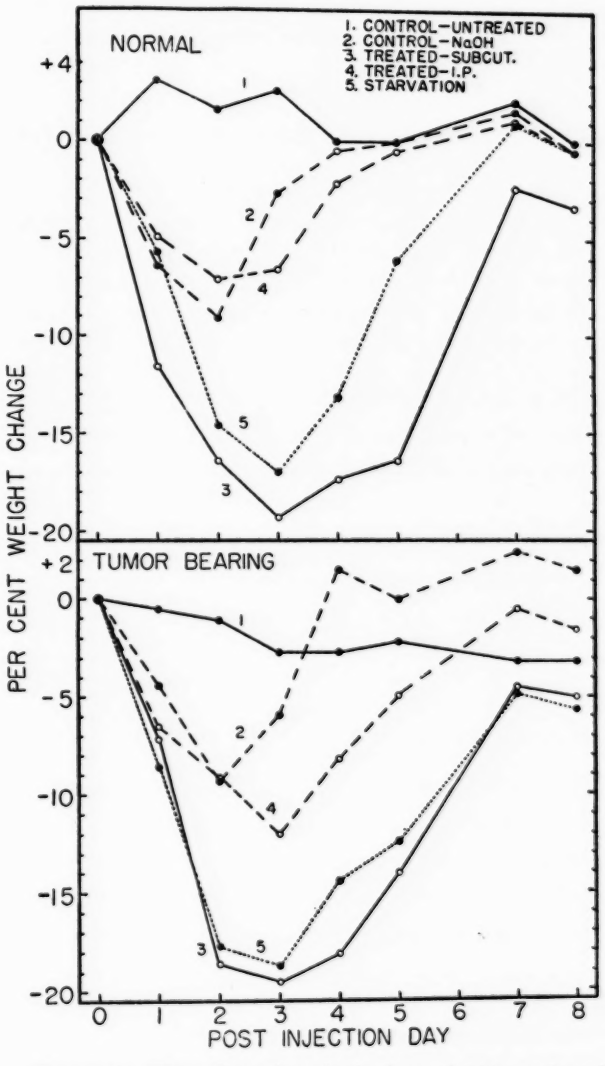


CHART 2.—The effect of a single dose of 500 mg/kg of 8-azaguanine, NaOH, and starvation on weight of normal and tumor-bearing mice; five mice in each group.

No gross changes were apparent in the stomach, intestines, pancreas, mesentery, kidneys, lung, liver, heart, or brain of 8-azaguanine-treated mice.

Morphological tumor changes.—Gross tumor changes were prominent in treated mice. Doses of 150 and 175 mg/kg/day produced a distinct diffuse pink color throughout the tumor mass, in contrast to the light color of the controls. The color was less in those mice that received 100 mg/kg/day. After doses of 175 mg/kg/day, there were also necrotic areas which were not grossly apparent in the controls of this group. Gross tumor damage was seen within 2–6 hours, with the most striking damage observed 24 hours after a single dose of 500 mg/kg. This was similar in appearance to that seen after a single effective dose of podophyllotoxin (11). The tumors were soft and diffusely hemorrhagic and often appeared completely hemorrhagic except for occasional islands of apparently unaffected tissue. This damage was apparently reversible, inasmuch as progressively less damage was observed on subsequent days.

TABLE 2

THE EFFECT OF A SINGLE DOSE OF 8-AZAGUANINE ON THE MITOTIC RATE OF SARCOMA 37 IN MICE*

	Control			8 Azaguanine		
Exp. no.	Cells/ hpf†	Mitoses/ hpf	Per cent mitoses	Cells/ hpf	Mitoses/ hpf	Per cent mitoses
1	792	9	1.14	521	0	0.00
	709	5	0.70	480	2	0.42
	722	4	0.55	428	1	0.23
	780	12	1.54	388	1	0.26
	787	8	1.02	387	2	0.52
Mean	758	7.6	0.99	441	1.2	0.29
2	763	10	1.31	428	0	0.00
	720	3	0.42	762	0	0.00
	772	4	0.52	409	0	0.00
	803	8	1.00	680	4	0.59
	782	8	1.02	467	0	0.00
Mean	768	6.6	0.85	549	0.8	0.12
3	970	15	1.55	595	0	0.00
	768	7	0.91	655	1	0.15
	848	7	0.82	413	0	0.00
	709	12	1.69	306	2	0.65
	744	7	0.94	654	1	0.15
Mean	808	9.6	1.18	525	0.8	0.19
4	791	12	1.52	809	2	0.25
	858	5	0.58	380	3	0.79
	797	7	0.88	614	2	0.32
	779	11	1.41	166	0	0.00
	785	10	1.27	572	0	0.00
Mean	802	9.0	1.13	508	1.4	0.27

* Tumor removed 24 hrs. after a single dose of 500 mg/kg; areas counted were chosen at random.

† hpf = high-power field.

The gross tumor damage and the inhibition of tumor growth by 8-azaguanine were closely associated with marked histological changes in the tumor. Examination of a tumor excised 24 hours after a single dose of 500 mg/kg (Figs. 1 and 2) revealed relatively few undamaged cells, but most of the tumor showed marked vascular damage, hemorrhage, and stasis along with pronounced cellular degeneration. Cytoplasmic damage was evidenced by eosinophilia, vacuolization, retraction, and disintegration. The changes observed in the nuclei were of several types: (*a*) pyknotic, clumped chromatin granules; (*b*) swollen, often fading nuclei, with disorganization or loss of distinct granules; (*c*) karyorrhexis, chromatin dust, nuclear debris; and (*d*) distortion of most of the visible mitoses. These cellular alterations are interpreted as representing various stages and possibly various types of degenerative processes. The mitotic figures included all stages, but were approximately one-fifth as frequent as in control tumors (Table 2). The number of cells was also reduced.

Forty-eight hours after a single dose of 8-azaguanine, cellular disintegration remained evident. However, the relative number of mitotic figures in viable areas had nearly returned to that of control tumors, thus paralleling the gross observations and indicating reversibility of 8-azaguanine action.

At the time of sacrifice, 24 hours after the last daily dose of 175 mg/kg/day, the tumors were of the same age as those described above, but were appreciably smaller. Histologically, they showed numerous necrotic foci, little hemorrhage, few distorted mitotic figures, and the mitotic number approached that of control tumors.

Tumors of mice that received daily doses of 100 mg/kg, which showed no appreciable inhibition in growth, revealed histological damage comparable to that in those mice which received the higher daily dose of the drug.

Tumor inhibition was not the result of malnutrition, since mice deprived of food and water showed a weight loss comparable to that seen after 8-azaguanine (Chart 2), but this was not accompanied by retarded tumor growth or morphological evidence of tumor damage.

Histological changes in the host.—No histological changes were observed in the spleen and the adrenal glands after a single dose of 500 mg/kg of 8-azaguanine. It will be recalled that spleens of mice that were treated daily were much smaller than control spleens, usually brown in color, and often translucent. A number of the adrenals were similarly discolored. On microscopic examination, the sinusoids in the spleen were not distinct, as if compressed, and there were comparatively few erythrocytes present. The cortex and medulla of the adrenal glands showed cytoplasmic vacuolization, and the inner zone of the fasciculata possessed large areas of degenerative cells. Similar

changes in the spleen and adrenal glands were observed in treated nontumor-bearing mice.

Representative tumors and spleens of mice that received the multiple injections of 8-azaguanine were employed in respiration studies in the Warburg apparatus and are reported in the following paper.

DISCUSSION

Inhibition of growth of Sarcoma 37 was observed after treatment with 8-azaguanine, in association with tumor damage and gross and histological signs of systemic toxicity. The discrepancy between these results and those of Greenberg *et al.* (7) and of Kidder *et al.* (9), who stated that they observed no response of this tumor to 8-azaguanine, is probably explained by a difference in dose level and/or a difference in the route of administration. Although an appreciable inhibition in growth with the lowest dose employed in this investigation (100 mg/kg) was not always observed, induced histological changes were evident.

A well defined separation of the tumor-inhibitory action of 8-azaguanine from the systemic effects of the drug was not apparent, and there was no appreciable difference between treated normal and tumor-bearing mice. These observations seem to indicate that the effects of the drug may result from a specific cytotoxic drug action and not necessarily from an exacerbation of a toxic or alarm reaction activated by the tumor, or that there may be a combination of a specific cytotoxic effect and an alarm reaction induced by 8-azaguanine. In experiments reported here it was observed that the administration of this drug caused anorexia, weight loss, and morphological changes in the spleen and adrenal gland, but other gross changes in vital organs were not observed. Sugiura *et al.* (16) observed extreme congestion and fine granules in the liver cytoplasm, extensive congestion in the kidneys, and a reduction of lymphoid tissue in the lymph nodes. Goldin *et al.* (6) reported that it produced systemic toxicity as seen in reduced food intake, weight loss, leukopenia, and death.

The gross observations suggest that there is no apparent accumulation of the drug in the body and that multiple injections of low doses do not produce a progressively increased gross effect on Sarcoma 37. The former observation is supported by the findings of Bennett *et al.* (2), who reported that approximately 92 per cent of the activity of injected 8-azaguanine-2-C^{14} was eliminated within 24 hours. Moreover, Mandel *et al.*[1] observed that approximately 50 per cent of the activity of injected 8-azaguanine-4-C^{14} was eliminated within 2 hours and approximately 98 per cent within 12 hours. It may be assumed, therefore, that a substantially effective dose must be employed from the outset in order to elicit a significant inhibition of growth of Sarcoma 37. This may be further supported by experiment 4b (Chart 1), in which an initial dose of 500 mg/kg was followed by daily doses of 100 mg/kg. This form of "vigorous" therapy produced "marked inhibition."

[1] In press.

The observations that 8-azaguanine produced extensive hemorrhage and stasis in the tumor mass may indicate that the necrosis is caused by an interference with the vascular supply, as has been proposed by Ludford (12) and by Algire *et al.* (1) for the mode of action of colchicine and bacterial polysaccharides. However, this does not appear to be a satisfactory explanation of the action of this compound, since other authors have not reported vascular degeneration or hemorrhage in tumors inhibited by 8-azaguanine, in those that showed induced necrosis, or in lymph nodes that were affected by this drug (16). Furthermore, in the present investigation, hemorrhage in spleens or adrenal glands, or an obstructed blood supply in the necrotic adrenals, was not observed.

SUMMARY

1. Data are presented on the effect of single and multiple doses of 8-azaguanine on growth, morphology, and drug toxicity in normal and tumor-bearing mice.

2. Multiple doses of 8-azaguanine produced a definite inhibition of growth and extreme cellular damage of Sarcoma 37.

3. Gross tumor damage was observed within 2–6 hours, with extensive morphological damage 24 hours after a single large dose of 8-azaguanine.

4. The number of mitotic figures in tumors after repeated doses of 8-azaguanine approached that of control tumors, whereas after a single large dose it was approximately one-fifth that seen in controls.

5. The drug was not completely destructive to this tumor. Progressively less effect on growth and morphology was seen on cessation of therapy.

6. No well defined separation of the tumor-inhibitory action of 8-azaguanine from systemic and morphological effects of the drug was apparent. The toxic action was similar in normal and tumor-bearing mice. Multiple doses produced morphological spleen and adrenal damage.

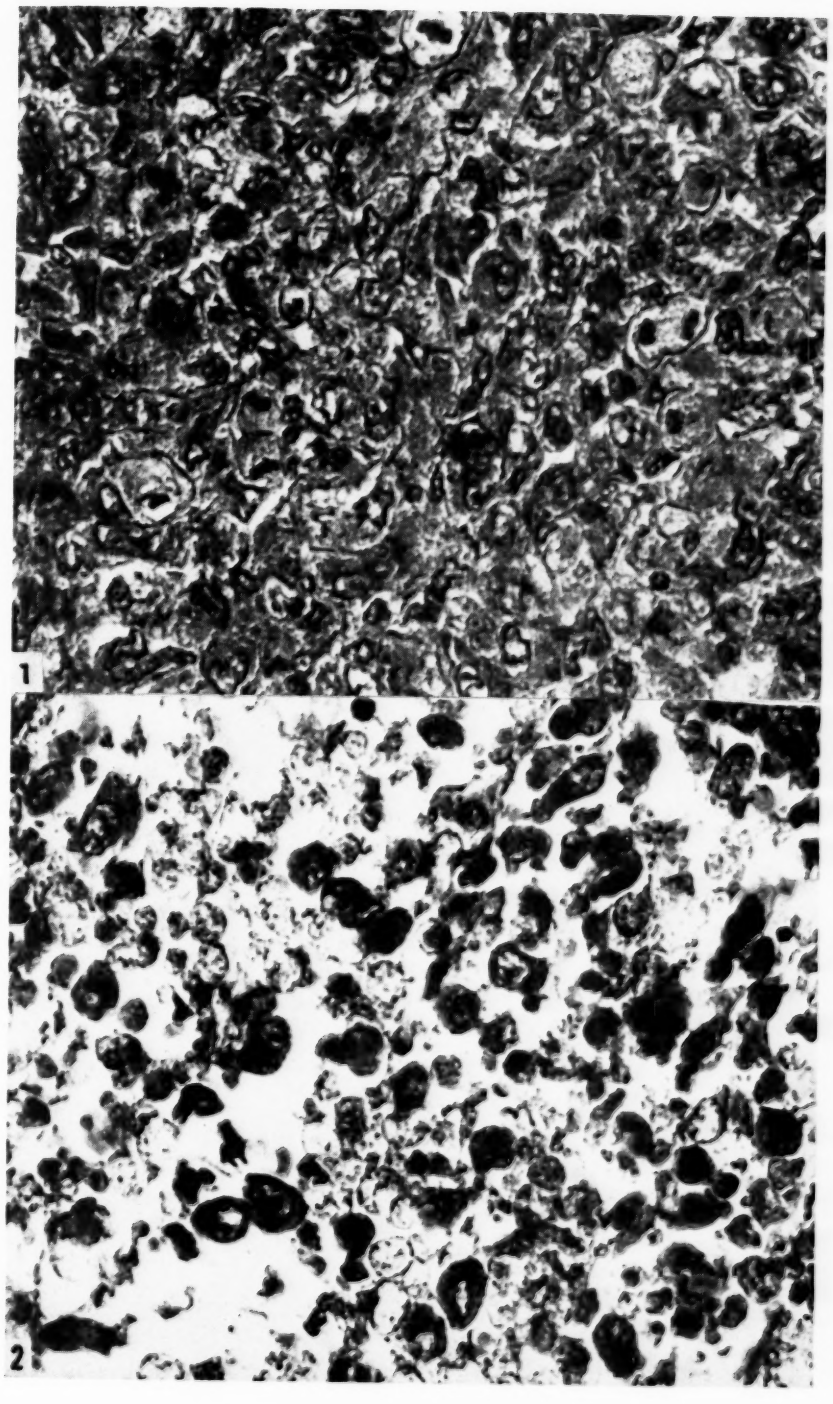
REFERENCES

1. ALGIRE, G. H.; LEGALLAIS, F. Y.; and PARK, H. D. Vascular Reactions of Normal and Malignant Tissues *in Vivo*. II. The Vascular Reactions of Normal and Neoplastic Tissues of Mice to a Bacterial Polysaccharide from *Serratia marcescens* (*Bacillus prodigiosus*) Culture Filtrates. J. Nat. Cancer Inst., **8**:53–62, 1947.

2. Bennett, L. L., Jr.; Skipper, H. E.; Mitchell, J. H., Jr.; and Sugiura, K. Studies on the Distribution of Radioactive 8-Azaguanine (Guanazolo) in Mice with E 0771 Tumors. Cancer Research, **10**:644–46, 1950.
3. Burchenal, J. H.; Johnston, S. F.; Burchenal, J. R.; Kushida, M. N.; Robinson, E.; and Stock, C. C. Chemotherapy of Leukemia. IV. Effect of Folic Acid Derivatives on Transplanted Mouse Leukemia. Proc. Soc. Exper. Biol. & Med., **71**:381–87, 1949.
4. Cavalieri, L. F.; Bendich, A.; Tinker, J. F.; and Brown, G. B. Ultraviolet Absorption Spectra of Purines, Pyrimidines and Triazolopyrimidines. J. Am. Chem. Soc., **70**:3875–80, 1948.
5. Gellhorn, A.; Engelman, M.; Shapiro, D.; Graff, S.; and Gillespie, H. The Effect of 5-Amino-7-Hydroxy-1*H*-*v*-Triazolo (*d*) Pyrimidine (Guanazolo) on a Variety of Neoplasms in Experimental Animals. Cancer Research, **10**:170–77, 1950.
6. Goldin, A.; Greenspan, E. M.; and Schoenbach, E. B. Studies on the Mechanism of Action of Chemotherapeutic Agents in Cancer. IV. Relationship of Guanine and Guanylic Acid to the Action of Guanazolo on Lymphoid Tumors in Mice and Rats. J. Nat. Cancer Inst., **11**:319–38, 1950.
7. Greenberg, D. M.; Irish, A. J.; and Gal, E. M. Effect of Metabolite Analogs on Tumor Growth of Transplantable Tumors. Cancer Research, **10**:221, 1950.
8. Kidder, G. W.; Dewey, V. C.; Parks, R. E., Jr.; and Woodside, G. L. Purine Metabolism in *Tetrahymena* and Its Relation to Malignant Cells in Mice. Science, **109**:511–14, 1949.
9. Kidder, G. W.; Dewey, V. C.; Parks, R. E., Jr.; and Gilbert, W. L. Further Evidence on the Mode of Action of 8-Azaguanine (Guanazolo) in Tumor Inhibition. Cancer Research, **11**:204–11, 1951.
10. Law, L. W. Studies on the Effect of Guanine Analog on Acute Lymphoid Leukemias of Mice. Cancer Research, **10**:186–90, 1950.
11. Leiter, J.; Downing, V.; Hartwell, J. L.; and Shear, M. J. Damage Induced in Sarcoma 37 with Podophyllin, Podyphyllotoxin, Alpha-Peltatin, Beta-Peltatin and Quercetin. J. Nat. Cancer Inst., **10**:1273–93, 1950.
12. Ludford, R. J. Colchicine in Experimental Chemotherapy of Cancer. J. Nat. Cancer Inst., **6**:89–101, 1945.
13. Roblin, R. O., Jr.; Lampen, J. O.; English, J. P.; Cole, Q. P.; and Vaughan, J. R., Jr. Studies in Chemotherapy. VIII. Methionine and Purine Antagonists and Their Relations to the Sulfonamides. J. Am. Chem. Soc., **67**:290–94, 1945.
14. Shapiro, D. M.; Weiss, R.; and Gellhorn, A. The Effect of Azaguanine on Mitosis in Normal and Neoplastic Tissue. Cancer, **3**:896–902, 1950.
15. Stock, C. C.; Cavalieri, L. F.; Hitchings, G. H.; and Buckley, S. M. A Test of Triazolopyrimidines on Mouse Sarcoma 180. Proc. Soc. Exper. Biol. & Med., **72**:565–67, 1949.
16. Sugiura, K.; Hitchings, G. H.; Cavalieri, L. F.; and Stock, C. C. The Effect of 8-Azaguanine on the Growth of Carcinoma, Sarcoma, Osteogenic Sarcoma, Lymphosarcoma and Melanoma in Animals. Cancer Research, **10**:178–85, 1950.

Fig. 1.—Sarcoma 37, untreated.

Fig. 2.—Sarcoma 37, 24 hours after a single dose of 500 mg/kg of 8-azaguanine.



The Effect of 8-Azaguanine on Tissue Metabolism in Mice Bearing Sarcoma 37*†

MURRAY FINKELSTEIN, WALLACE D. WINTERS, PAUL A. THOMAS, CLARKE DAVISON, AND PAUL K. SMITH

(Department of Pharmacology, the George Washington University School of Medicine, Washington, D.C.)

The observation of Kidder and his associates (4) that 8-azaguanine selectively inhibits the development of a number of malignant neoplasms supports the view that there exists a biochemical differentiation between normal and neoplastic cells. Despite the interesting biological properties of 8-azaguanine, little attention has thus far been given to the effect of this compound on tissue metabolism. Earlier studies from this laboratory have shown that another carcinostatic compound, podophyllotoxin, selectively inhibits the respiration of tumor slices from mice (7). The present report is an attempt to determine the mode of action of 8-azaguanine and involves an examination of the effect of *in vitro* and *in vivo* administration of this compound on the respiration of tissues of CAF_1 mice bearing Sarcoma 37.

MATERIALS AND METHODS

The respiration of tissues of CAF_1 mice, weighing 18–22 gm., bearing Sarcoma 37, was measured by the standard Warburg manometric technic, at 38° C. The medium employed for tissue slices consisted of a modified Krebs-Ringer phosphate solution with the following concentration of components: NaCl, 123 mM; KCl, 4.5 mM; $CaCl_2$, 0.86 mM; $MgSO_4$, 12.4 mM; sodium phosphate buffer, 20 mM at pH 7.35; and glucose, 11.1 mM per liter. Tissue homogenates were prepared in phosphate buffer, 20 mM per liter at pH 7.35. Preliminary studies showed that the presence or absence of glucose as the substrate in the medium did not alter the rate of oxygen consumption of control tumor slices or those that were respiring in the presence of 8-azaguanine. Consequently, glucose was employed in the medium in all subsequent experiments. Other preliminary experiments showed that there was no difference between the oxygen uptake of control mice that received subcutaneous injections of NaOH, in concentrations similar to those employed with the drug, and that of control mice that received no injection.

Tumor age varied from 5 to 6 days at the time of parenteral administration of 8-azaguanine and from 6 to 7 days when the drug was added directly to respiring tissues. In the former procedure, the drug was dissolved in NaOH, administered in single subcutaneous injections (as described previously[1]), and the mice were sacrificed at various time intervals after injection; in the latter situation, 8-azaguanine was employed as a 0.025–0.1 per cent water suspension. The respiration of tumors and spleens of mice that received multiple doses of the drug was also examined. The effect of 8-azaguanine on the growth and morphology of these tissues was described earlier.[1]

Respiration was measured for at least 1 hour, and readings were taken every 15 minutes. However, with the exception of Table 2, the oxygen consumption is expressed as the oxygen uptake per hour in cubic millimeters per 100 milligrams wet weight of tissue. Where desirable, the *t* test was employed, and P value was obtained as an index of the significance of observed differences in experiments.

Several experiments were also carried out on Sarcoma 37 in C strain mice, and the results were essentially the same as those obtained in the CAF_1 strain. In a number of experiments 8-azaguanine was administered intraperitoneally, and the results were substantially similar to those described here. The data obtained from these groups of experiments are not included in this report.

RESULTS

In vitro experiments.—The addition of 8-azaguanine in a water suspension in doses of 0.05,

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[1] See preceding paper in this issue.

0.1, and 0.2 mg/100 mg of tumor (final concentrations of 2.5, 5.0, and 10 mg/100 cc, respectively) 1 hour after the beginning of measured respiration did not affect the oxygen consumption of slices of Sarcoma 37. Similarly, a dose of 0.2 mg/100 mg of tissue did not alter the oxygen uptake of spleen, liver, or kidney slices. Table 1 shows the results of

TABLE 1

EFFECT OF 8-AZAGUANINE *in Vitro* ON RATE OF RESPIRATION OF MOUSE TISSUE SLICES*

PERIOD AFTER START OF EXPERIMENT	TUMOR		KIDNEY		LIVER		SPLEEN	
	(mm³/100 mg wet tissue)							
1st hr.	86	84	394	395	92	93	150	154
	Control	Drug	Control	Drug	Control	Drug	Control	Drug
2d hr.	84	82	400	407	92	90	141	146
3d hr.	80	78	399	402	89	91	141	143
4th hr.	80	79	396	398	88	90	135	138

* Readings were taken every 15 minutes; dose, 0.2 mg/100 mg of tissue.

a typical experiment with the highest drug concentration only. In another set of experiments the drug was added at the beginning of measured respiration and was without effect. Similarly, the oxygen uptake in experiments employing tumor homogenates was not influenced by 8-azaguanine.

Effect of single doses of 8-azaguanine.—Preliminary *in vivo* experiments indicated that 8-azaguanine produced an inhibition of oxygen uptake of tumor slices; consequently, the relationship between drug dosage and the degree of inhibition of tumor respiration was determined. The mice were sacrificed 24 hours after drug injection, and the results (total of 82 mice) are summarized in Chart 1. It is interesting to note that it was generally possible to predict in advance whether or not there would be an inhibition in the rate of respiration from the gross appearance of the tumor, i.e., softening and diffuse hemorrhages of the tumor mass. The rate of and total oxygen consumption of tumor slices was essentially unaltered after doses up to 150 mg/kg; doses of 200 and 250 mg/kg produced an inhibition of approximately 21 per cent, which was barely significant statistically. With progressively larger doses there was a correspondingly increased effect, with almost complete inhibition (approximately 92 per cent) after 500 mg/kg, the highest dose employed.

Since the most effective dose in 24 hours was 500 mg/kg and this also produced significant gross and histological tumor changes in this short period, this dose was employed in the following experiments.

The results of the effect of a single dose of 500 mg/kg of 8-azaguanine on tumors of 209 mice, sacrificed at various time intervals after the injections, are summarized in Chart 2. It can be seen that there was no appreciable effect 1 hour after drug administration; however, beginning with tumors of mice sacrificed 2 hours, and in those up to 24 hours, after drug administration, there was a progressively decreased oxygen uptake (42 per cent and up to 92 per cent inhibition, respectively). Forty-eight hours after a single dose the effect was less (approximately 26 per cent inhibition) with a relatively large range but, nevertheless, statistically significant (probability < 0.01). The oxygen uptake of kidney, spleen, liver, and brain slices of a total of 134 mice, whose tumor respiration was inhibited by a single dose of 500 mg/kg of the drug, was essentially unaffected. Any differences from the controls were statistically insignificant.

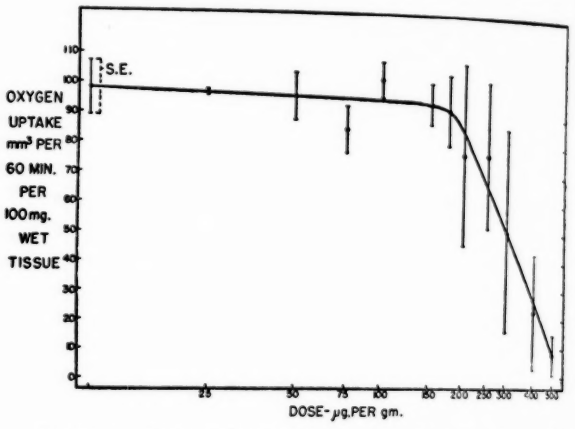

CHART 1.—Dose response of Sarcoma 37 to 8-azaguanine. Mice were sacrificed 24 hours after single subcutaneous injection.

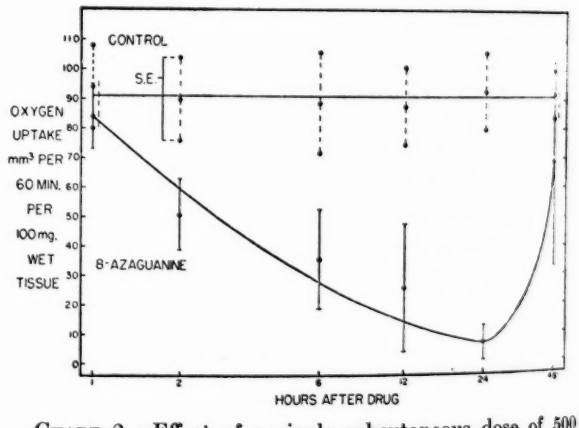

CHART 2.—Effect of a single subcutaneous dose of 500 mg/kg of 8-azaguanine on tumor respiration. Mice were sacrificed at various time intervals after drug injection.

The observation that the spleen was diminished

in size[1] after administration of 8-azaguanine suggested that this organ might be necessary for the action of the drug. Respiration studies were made on tumors of 8-azaguanine-treated splenectomized mice. In appropriately controlled experiments this procedure was without effect on the degree of inhibition of tumor respiration caused by the drug. In another set of experiments, spleen, liver, and kidney were homogenized and incubated for 2 hours in the presence of 8-azaguanine. These were then centrifuged, and the supernatant fluid was added to tumor slices at the beginning of and during measured respiration; these were observed with appropriate controls. There was no alteration in the rate of oxygen uptake of tumor slices during the subsequent 2 hours of observation.

It has been demonstrated by several investigators that the effect of 8-azaguanine on growth of unicellular organisms (3, 8) and on certain neoplasms (1, 5, 6) could be reversed by the administration of appropriate quantities of guanine. In an attempt to verify this with respect to the effect on oxygen consumption of slices of Sarcoma 37, doses of guanine equal to and up to 3 times (1,500 mg/kg) the dose of 8-azaguanine were injected either simultaneously with or 4 hours prior to the administration of the guanine analogue. This did not affect the inhibition of respiration produced by 8-azaguanine. It is very likely, as was indicated by Kidder (4) and as has been shown by others (1, 5, 6), that massive doses of guanine are required in order to overcome the activity of 8-azaguanine. Larger doses of guanine than those reported here were not employed, because of the low solubility of this compound and the toxicity of the drug to the host if it is used in larger amounts.

Effect of multiple doses of 8-azaguanine.—The effect of 8-azaguanine on the morphology of the tissues employed in this series of experiments was described previously.[1] The average results of a total of 33 mice (13 of these in duplicate) are summarized in Table 2. Five doses of 100 mg/kg/day and four doses of 150 mg/kg/day produced statistically significant inhibition (probability $\ll 0.02$ and < 0.01) of approximately 22 and 16 per cent, respectively, in the total oxygen uptake of tumor slices. Similar doses produced relatively consistent though statistically insignificant effects (probability < 0.1) on the oxygen consumption of spleens of these mice. However, when an initial dose of 500 mg/kg was followed by five doses of 100 mg/kg/day, there was a statistically significant inhibition (probability $\ll 0.02$) of approximately 15 per cent in the total oxygen uptake of the spleens.

DISCUSSION

The absence of an effect of 8-azaguanine on the respiration of mouse tissues other than tumor suggests the specificity of the drug for tumor tissue and supports the hypothesis of Greenstein (2) that a fundamental qualitative biochemical deviation from normal tissue exists in tumors. It appears unlikely that the specificity of the drug for Sarcoma 37 is caused by the rapidly proliferating nature of the tumor, in view of the observations of Shapiro *et al.* (9) that 8-azaguanine affected the mitotic index of tumor tissue while not that of the testis and intestinal epithelium of the host.

The action of the injected drug is apparently not mediated through the spleen or adrenal glands, since, as has been demonstrated here, the effect on tumor respiration was not altered by splenectomy; and Law (6) demonstrated that 8-azaguanine was effective in reducing the rate of growth of leukemic cells in adrenalectomized mice.

Kidder *et al.* (5) suggested that the action of the drug is not primarily on the host tissues, since not all tumors are affected. In support of this, they cite the report of Sugiura *et al.* (11), who observed that 8-azaguanine had no inhibitory effect on Sarcoma 180, even when the tumor was transplanted into the normal host for E 0771 (which is inhibited by the drug). In the present investigation, no effect on tumor respiration was shown when the

TABLE 2

EFFECT OF REPEATED DOSES OF 8-AZAGUANINE ON THE RATE OF RESPIRATION OF SARCOMA 37 AND SPLEEN SLICES*

Period after start of experiment (minutes)	Tumor		Spleen			Tumor		Spleen	
	Control	Drug (5×100 mg/kg)	Control	Drug (6×100 mg/kg)	Drug (1×500 mg/kg† +5×100 mg/kg)	Control	Drug (4×150 mg/kg)	Control	Drug (4×150 mg/kg)
0–15	19	14	37	35	31	23	20	41	38
15–30	20	17	39	36	33	23	18	42	37
30–45	18	14	39	36	33	23	20	39	36
45–60	20	15	37	34	32	23	19	40	38

* Tumors and spleens removed 24 hours after final dose. Results expressed in mm^3 O_2 uptake/100 mg wet tissue.
† An initial dose of 500 mg/kg followed by daily doses of 100 mg/kg.

drug was added *in vitro*. In this case, the tumor may be utilizing its intermediary metabolites without oxygen as a hydrogen acceptor. In experiments after drug administration, the effect on the tumor may have been mediated through some mechanism outside the tumor mass, or it is possible that a metabolic product of 8-azaguanine possesses the carcinostatic power. Mandel *et al.*[2] have demonstrated that at least 90–95 per cent of the radioactive urinary metabolites of administered 8-azaguanine-2-C^{14} or -4-C^{14} was recovered from either normal or tumor-bearing mice in the form of 8-azaxanthine. Sugiura *et al.* (11) used a sample of 8-azaguanine that contained 60 per cent 8-azaxanthine, and their results indicate that it was less effective on tumor growth than 8-azaguanine with a high degree of purity.

Mandel *et al.*[2] observed that approximately 50 per cent of injected 8-azaguanine-4-C^{14} (325 mg/kg) was eliminated within 2 hours and approximately 98 per cent within 12 hours. The maximum inhibitory effect on tumor respiration was observed 24 hours after drug injection. This corresponds to the nearly complete destruction of tumor tissue observed at this time.[1] The maximal effect on tumor respiration occurring 12 hours after drug elimination may be caused by the destruction of an enzyme system or its site of formation. With tumor destruction there is a progressive decrease in respiration. The diminished inhibition 36 hours after drug elimination corresponds to resumption of tumor growth and may indicate that there is a regeneration of this enzyme system, resulting in an increased respiration. By reducing the effective concentration of the enzyme(s), an inhibition of nucleic acid formation may result. Recently, Skipper *et al.* (10) reported that 8-azaguanine inhibited nucleic acid synthesis *in vivo*. Since rapidly growing tissue, such as Sarcoma 37, would require a greater concentration of the nucleic acids, growth would be reduced.

There is an obvious correlation between tumor damage, with a reduction in mitosis, and the parallel reduction in oxygen uptake. The question exists whether the primary effect of 8-azaguanine is to block the respiratory processes, and in this way inhibit growth, or whether it prevents the synthesis of an essential metabolite or competes with it for a given enzymic system, thus affecting respiration only indirectly.

SUMMARY

1. Data are presented on the effect of 8-azaguanine on the respiration of tissues of CAF_1 mice bearing Sarcoma 37.

2. There was no effect on the respiration of tumor homogenate and tumor, brain, liver, kidney, or spleen slices when the drug was added directly to the respiring tissues. The addition of the supernatant fluid from homogenates of spleen, liver, or kidney incubated with the drug was similarly without effect on the respiration of tumor slices.

3. 8-Azaguanine showed a selective action on tumor tissue after parenteral administration. The minimal effective dose was 200 mg/kg. Two hours after a single dose of 500 mg/kg there was a significant inhibition of respiration, which became almost complete after 24 hours. Forty-eight hours after drug administration, the effect on tumor respiration was diminished.

4. Reversal of 8-azaguanine action was not produced by the administration of guanine.

5. The possible significance of these results has been discussed.

REFERENCES

1. Goldin, A.; Greenspan, E. M.; and Schoenbach, E. B. Studies on the Mechanism of Action of Chemotherapeutic Agents in Cancer. IV. Relationship of Guanine and Guanylic Acid to the Action of Guanazolo on Lymphoid Tumors in Mice and Rats. J. Nat. Cancer Inst., **11**:319–38, 1950.
2. Greenstein, J. P. Biochemistry of Cancer. New York: Academic Press, Inc., 1947.
3. Kidder, G. W., and Dewey, V. C. The Biological Activity of Substituted Purines. J. Biol. Chem., **179**:181–87, 1949.
4. Kidder, G. W.; Dewey, V. C.; Parks, R. E., Jr.; and Woodside, G. L. Purine Metabolism in *Tetrahymena* and Its Relation to Malignant Cells in Mice. Science, **109**:511–14, 1949.
5. Kidder, G. W.; Dewey, V. C.; Parks, R. E., Jr.; and Gilbert, W. L. Further Evidence on the Mode of Action of 8-Azaguanine (Guanazolo) in Tumor Inhibition. Cancer Research, **11**:204–11, 1951.
6. Law, L. W. Studies on the Effects of a Guanine Analog on Acute Lymphoid Leukemias of Mice. Cancer Research, **10**:186–90, 1950.
7. Miller, Z. B.; Davison, C.; and Smith, P. K. The effect of Podophyllotoxin on Tissue Metabolism and Enzyme Systems. J. Exper. Med., **90**:525–41, 1949.
8. Roblin, R. O., Jr.; Lampen, J. O.; English, J. P.; Cole, Q. P.; and Vaughan, J. R., Jr. Studies in Chemotherapy. VIII. Methionine and Purine Antagonists and Their Relations to the Sulfonamides. J. Am. Chem. Soc., **67**:290–94, 1945.
9. Shapiro, D. M.; Weiss, R.; and Gellhorn, A. The Effect of Azaguanine on Mitosis in Normal and Neoplastic Tissue. Cancer, **3**:896–902, 1950.
10. Skipper, H. E.; Mitchell, J. H., Jr.; Bennett, L. L., Jr.; Newton, M. A.; Simpson, L.; and Eidson, M. Observations on Inhibition of Nucleic Acid Synthesis Resulting from Administration of Nitrogen Mustard, Urethan, 2,6-Diaminopurine, 8-Azaguanine, Potassium Arsenite, and Cortisone. Cancer Research, **11**:145–49, 1951.
11. Sugiura, K.; Hitchings, G. H.; Cavalieri, L. F.; and Stock, C. C. The Effect of 8-Azaguanine on the Growth of Carcinoma, Sarcoma, Osteogenic Sarcoma, Lymphosarcoma and Melanoma in Animals. Cancer Research, **10**:178–85, 1950.

[2] In press.

The Significance of the Anterior Chamber in Tumor Transplantation

II. The Nature of Tumor Growth beyond the Anterior Chamber*

E. J. Eichwald and H. Y. Chang

(*Department of Pathology, University of Utah, College of Medicine, Salt Lake City, Utah*)

When mouse neuroblastoma C1300 was transplanted, in this laboratory, into the anterior chamber of the eye of female mice of the C57 brown strain (subline cd), it grew progressively in 152 of 199 animals (76.4 per cent). Following transplantation to the subcutaneous tissue of the abdominal wall, the tumor grew in only 42 of 216 mice of this strain (19.4 per cent). When transplanted to the anterior chamber, these tumors extended beyond the eye and infiltrated the periocular tissue and subcutaneous tissues of the head of all animals in which the tumors grew progressively. Death occurred within similar intervals in the two groups. The periocular tissue and the subcutaneous tissue of the head of the majority of animals were therefore susceptible to invasion by the tumor following its inoculation into the anterior chamber.

In an attempt to interpret this observation, the following possibilities were considered: (*a*) The resistance to the tumor in the periocular tissue and cranial subcutaneous tissue was lower than that of the abdominal subcutaneous tissue. (*b*) While growing in the anterior chamber the tumor had adapted itself to the strain or to the individual host. (*c*) The subcutaneous tissue of the mice was resistant only to the newly implanted tumor, but not to the tumor already established in a less resistant site.

The experiments reported below were devised to determine whether the periocular tissue differs from the abdominal subcutaneous tissue in its susceptibility to the tumor under study and whether, during the period of anterior chamber growth, the tumor adapts itself to the strain of mice or to the individual host.

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MATERIALS AND METHODS

Neuroblastoma C1300, obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, has been carried in our laboratory for 34 transplant generations by abdominal subcutaneous transplantation in ABC mice. Female mice of the C57 brown strain (subline cd), weighing 15 gm. or more at the time of inoculation, also were obtained from Bar Harbor. The mice were fed a commercial diet and water *ad libitum*.

The tumors were transplanted to the anterior chamber following the method described previously (1). Transplantation to the periocular tissue was achieved as follows: (*a*) The conjunctival mucosa was perforated at the inner canthus, and the tip of the trocar was directed horizontally underneath the mucosa to the vicinity of the outer canthus. Detachment of the graft from the needle was assured by pinching the overlying mucosa on withdrawing the trocar. (*b*) The cranial subcutaneous tissue was inoculated by nicking the scalp in the midline, and by directing the trocar laterally, to deposit the graft in the vicinity of the eye. The inoculations in all four groups of the first experiment were done with a No. 20 spinal needle under ether anesthesia.

Tumor tissue for homologous retransplantation was obtained by reflecting the scalp anteriorly over the cranial tumor, removing a portion of the tumor, and re-inoculating this subcutaneously with a No. 17 spinal needle.

Tumor tissue for autologous retransplantation on the eighth and eleventh days after transplantation was obtained by incising the cornea along the limbus and shelling the tumor out of the anterior chamber. At this time, growing tumor was found in all inoculated mice of this strain.

On the 18th and 27th days tumor tissue was obtained by stripping back the upper lid over the

bulging tumor and incising the visceral leaf of the conjunctiva. It was then retransplanted to the abdominal subcutaneous tissue with a No. 17 spinal needle. The validity of these transplantation procedures with respect to the transfer of viable tumor cells was ascertained by inoculating mice of the ABC strain, which are highly susceptible to this tumor.

EXPERIMENTAL

Susceptibility of extra-ocular cranial tissues.—Neuroblastoma C1300 was transplanted to the anterior chamber of 82 mice, to the abdominal subcutaneous tissue of 84 mice, to the conjunctival tissue of 92 mice, and to the cranial subcutaneous tissue of 81 mice. The data in Table 1

TABLE 1

SUSCEPTIBILITY OF EXTRA-OCULAR CRANIAL TISSUES OF C57 BROWN MICE TO MOUSE NEUROBLASTOMA C1300

Transplantation site	Incidence of tumor growth	Per cent
Conjunctiva	24/92	26
Cranial subcutaneous tissue	17/81	21
Subcutaneous tissue	15/84	18
Anterior chamber	68/82	83

show that there was no significant difference in susceptibility to this tumor among the conjunctival, the cranial, and the abdominal subcutaneous group. The difference between each of these groups and the anterior chamber group was significant. It indicates that in mice of this strain the periocular and cranial connective tissue are not more susceptible to neuroblastoma C1300 than the tissue of the abdominal wall.

Adaptation of tumor cells to homologous hosts.—Neuroblastoma C1300 was transplanted to the anterior chamber of nine mice and to the subcutaneous tissue of the abdomen of twenty mice. The tumor grew progressively in all mice which had received inoculations in the anterior chamber, and in four of the subcutaneously inoculated mice. Thirty-seven days after inoculation seven mice of the anterior chamber group and three mice with growing tumors of the subcutaneous group were sacrificed for retransplantation of the tumor. The animals selected had large tumors and were agonal. Fragments of each tumor were retransplanted to the abdominal subcutaneous tissue of ten mice. Sixty-one days after transplantation a mouse from the anterior chamber group and one from the original subcutaneous group were sacrificed, and the tumors were retransplanted to the abdominal subcutaneous tissue of nine mice. The last mouse of the anterior chamber group was sacrificed on the 73d day and the tumor retransplanted to ten mice.

Retransplantation of the tumor to the abdominal subcutaneous tissue resulted in progressive growth in 4 of 89 mice (4.5 per cent) following anterior chamber inoculation, and in 4 of 39 mice (10.3 per cent) following subcutaneous inoculation (Table 2). These data indicate that adaptation of the tumor to hosts of the same strain is not the cause of its growth beyond the eye following transplantation to the anterior chamber.

Adaptation of tumor cells to autologous hosts.—Neuroblastoma C1300 was transplanted to the anterior chamber of 122 mice and to 55 subcutaneous controls. Eleven mice of the subcutaneous group developed progressively growing tumors (20 per cent). Fifty-eight mice of the anterior chamber group served as controls; 42 of these (72 per cent) developed progressively growing tumors. The remaining mice of the anterior chamber group were set aside for autologous retransplantation. Of these, tumors were removed from the anterior chambers of fifteen on the 8th day and of

TABLE 2

ADAPTATION OF MOUSE NEUROBLASTOMA C1300 TO HOMOLOGOUS HOSTS (C57 BROWN)

Site of original transplantation	Day of retransplantation	Incidence of tumor growth following subcu. retransplantation
Anterior chamber		
No. 1	37	0/10
" 2	37	0/10
" 3	37	1/10
" 4	37	1/10
" 5	37	2/10
" 6	37	0/10
" 7	37	0/10
" 8	61	0/10
" 9	73	0/9
Subcutaneous tissue		
No. 1	37	2/10
" 2	37	0/10
" 3	37	1/10
" 4	61	1/9

twenty on the 11th day after transplantation. In twenty mice, tumors were removed from the extra-ocular cranial tissue on the 18th day, and in six mice on the 27th day after transplantation. In each animal the tumor was retransplanted to the abdominal subcutaneous tissue. In the three remaining mice the tumor failed to grow progressively. Tumor growth following autologous retransplantation to the abdominal wall was observed in only 1 of the 61 mice (18th-day group) (Table 3). These data indicate that autologous

adaptation is not the cause for the observed tumor growth beyond the anterior chamber.

CONCLUSIONS

When transplanted to the anterior chamber of the eye, mouse neuroblastoma C1300 grows progressively and invades the cranial subcutaneous tissue in the majority of female mice of the C57 brown strain. Following transplantation to the abdominal subcutaneous tissue, it fails to grow in the majority of mice of this strain.

TABLE 3

ADAPTATION OF MOUSE NEUROBLASTOMA C1300 TO AUTOLOGOUS HOSTS (C57 BROWN) FOLLOWING TRANSPLANTATION TO ANTERIOR CHAMBER

No. of mice	Day of retrans-plantation	Incidence of tumor growth following subcu. retrans-plantation
15	8	0
20	11	0
20	18	1
6	27	0

The experiments reported show that progressive growth of the tumor beyond the anterior chamber and beyond the eye is not due to adaptation of the tumor to this strain of mice and not due to adaptation to the individual host. Neither do the periocular and cranial subcutaneous tissues exceed the abdominal subcutaneous tissue in susceptibility to this tumor.

It appears that a tumor can invade the subcutaneous tissue of a host following its establishment in the anterior chamber, even if the same tumor will fail to grow when transplanted subcutaneously. The significance of the anterior chamber, as compared to the subcutaneous tissue, then, lies in the fact that within its confines the resistance to the initial growth of a tumor is low. Once the tumor has become established, it can overcome the resistance of the subcutaneous tissue, and grow as well outside as within the anterior chamber. It follows that the low resistance in the anterior chamber is essential only to the growth of the newly transplanted tumor to which the subcutaneous tissue is resistant.

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REFERENCES

1. EICHWALD, E. J.; EVANS, R. G.; and BROWNING, G. B. The Significance of the Anterior Chamber in Tumor Transplantation. I. Transplantation of Mouse Neuroblastoma C1300 to Homologous Hosts. Cancer Research, **10**:483–85, 1950.

A Sex Difference in the Development of Liver Tumors in Rats Fed 3′-Methyl-4-dimethylaminoazobenzene or 4′-Fluoro-4-dimethylaminoazobenzene*

H. W. Rumsfeld, Jr., W. L. Miller, Jr., and C. A. Baumann

(Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison 6, Wis.)

INTRODUCTION

Sex differences in the response of rats to the carcinogen 2-acetylaminofluorene (AAF) are well known (1, 10, 20). Liver tumors develop readily in males fed this carcinogen, while females are more resistant to hepatoma development. On the other hand, no marked sex differences have been reported in the response of rats to the azo dyes. The dye usually fed has been 4-dimethylaminoazobenzene (DAB), but since 1945 the more potent 3′-methyl-DAB (also referred to as *m*′-methyl-DAB) has also been fed extensively. In certain of these latter studies (4, 14), data have been accumulating which suggest that males develop liver tumors more readily than females when low doses of 3′-Me-DAB are fed continuously or high doses are fed intermittently. Accordingly, the effect of sex on tumors due to azo dyes has now been studied in more carefully controlled experiments. Young male and female rats from the same source[1] were raised to the proper starting weight on a synthetic diet prior to the feeding of 3′-Me-DAB or 4′-fluoro-4-dimethylaminoazobenzene (4′-F-DAB) in amounts appropriate for moderate tumor development. 3′-Me-DAB was also fed to castrate male and ovariectomized female rats, while in parallel studies measurements were made of the ability of liver slices or homogenates from male and female rats to destroy the carcinogens DAB, 3′-Me-DAB, or 4′-F-DAB *in vitro*.

METHODS

Tumor production.—Equal numbers of male and female rats of the same age and ranging from 80 to 110 gm. in weight were kept in wire-bottomed cages in groups of seven or eight. Fifteen males were castrated and fifteen females ovariectomized and allowed to recover fully before any carcinogen was fed. All groups were fed a relatively complete synthetic diet (I) which consisted of extracted[2] casein, 18; salts, 4; corn oil, 5; and glucose monohydrate (Cerelose) to 100, with vitamins added at the following levels in mg/kg diet: thiamine, 2; pyridoxine, 2.5; riboflavin, 3; niacin, 10; calcium pantothenate, 20; inositol, 100; biotin, 0.1; folic acid, 0.2; *p*-aminobenzoic acid, 125; cystine, 1,000; and choline chloride, 1,000. Vitamins A and D were supplied by 2 drops of halibut liver oil per rat every 4 weeks.

When the males reached approximately 200 gm. in weight and the females approximately 180 gm., the rats were divided into groups and fed diet II, which is known to favor the development of hepatomas (6, 15, 18). It consisted of extracted casein, 12; salts, 4; corn oil, 5; and glucose monohydrate to 100 with vitamins added at the following levels in mg/kg diet; thiamine, 3; pyridoxine, 2.5; calcium pantothenate, 7.5; riboflavin, 2; and choline chloride, 1,000. Each rat also received 2 drops of halibut liver oil every 4 weeks.

The carcinogens 3′-Me-DAB and 4′-F-DAB[3] were incorporated into the diets at various levels. When the dyes were fed continuously, the level in the diet was 0.040 per cent; for experiments in which the dye was fed intermittently, the level was 0.064 per cent. In the initial experiments (Tables 1 and 3, groups 1–4), the dye was usually fed for 4 weeks, followed by 4 weeks of the dye-free basal ration with or without further alterations in diet, after which the ration containing dye was

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[1] Holtzman-Rolfsmeyer Co., Madison, Wis.

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[2] Forty pounds of crude commercial casein are extracted once with 18 gallons of 95 per cent ethanol for 1½ hours at 70° C., and twice more with 10 gallons of alcohol. The casein is filtered after each extraction, and after the final filtration it is dried in a stream of air at 45°–55° C.

[3] Supplied by Dr. J. A. Miller of the McArdle Memorial Laboratory of this university.

fed for 4 more weeks. Essentially the same procedure was followed in the later, more carefully controlled experiments (Table 2, groups 1–4, and Table 3, groups 5–8), with an additional week of dye in the final period. In other series (Table 2, groups 5–10), the dye was fed continuously for 12 or 14 weeks. After the completion of the dye-feeding period, the animals were continued on the basal diet free from dye for at least 12 weeks longer to permit any hepatomas induced by the carcinogen to grow to recognizable size. In some series the basal diet was fed for an even longer period of time.

Dye destruction in vitro.—Weanling rats[1] of both sexes were fed diet III, which was like diet II except that the casein level was increased to 18 per cent at the expense of glucose, and the riboflavin was increased to 10 mg/kg. After 2 weeks on diet III the animals of each sex were divided into three groups. One group was fed diet III, another was fed diet IV, which was like diet III except that the riboflavin was lowered to 2 mg/kg, while a third group was fed diet IV with 0.04 per cent of 3′-Me-DAB added. Rats were sacrificed at various intervals, and liver slices as well as homogenates were prepared and incubated with DAB, 3′-Me-DAB, or 4′-F-DAB, as in previous studies on the destruction of DAB *in vitro* (14, 16). For the slice system each incubation flask contained 200 mg. of liver slices (wet), 1.9 ml. of Krebs-Ringer phosphate buffer, adjusted to pH 7.4, and 100 μg. of dye. After 1 hour of incubation at 37° C., the remaining dye was recovered and determined colorimetrically in 7 N HCl with the 515 mμ Evelyn filter. The K_{515} used in the expression $C = KL$ was determined for DAB, 3′-Me-DAB, and 4′-F-DAB, and found to be 29.2, 31.6, and 42.8, respectively. The homogenate system was that described previously (14, 16).

RESULTS

Table 1 indicates the development of hepatic tumors in all rats fed 3′-Me-DAB in this laboratory during 1945–51 in experimental series in which both male and female rats were used. The incidence of liver tumors was higher in the males than in the females for each type of experiment summarized, and the total tumor incidences were 187/342 or 55 per cent for the males, as compared to 49/314 or 16 per cent for the females. This result was interpreted to suggest rather than to demonstrate a sex effect in the response of rats to 3′-Me-DAB, since the representatives of the two sexes were not necessarily of the same age, nor from the same source, nor did they always have the same dietary history. Thus, in a given series the males may have been grown to maturity on synthetic rations, whereas the females were from our stock colony, or either or both may have come directly from the dealer without assurance of comparable history. However, since the number of groups was large and the results were consistent, it would appear that the observed differences in tumor incidence were primarily due to sex rather than to other factors.

In more controlled experiments (Table 2), the sex differences in tumor incidence were again pronounced. When 3′-Me-DAB was fed continuously for 12 weeks (Table 2, groups 5 and 6), the tumor incidence in the males was 5/15, while no tumors

TABLE 1

THE INFLUENCE OF SEX ON THE DEVELOPMENT OF LIVER TUMORS IN RATS FED 0.064 PER CENT 3′-METHYL-DAB (COMPOSITE DATA*)

		Male		Female	
Group	Method of dye feeding	Incidence of tumors	Per cent tumors	Incidence of tumors	Per cent tumors
A	3′-Me-DAB 4 wks.; basal diet 4 wks.; 3′-Me-DAB 4 wks.; basal diet at least 8 wks.	34/66	52	2/66	3
B	3′-Me-DAB 4 wks.; varied diets† 4 wks.; 3′-Me-DAB 4 wks.; basal diet at least 8 wks.	103/181	57	33/177	19
C	3′-Me-DAB 6 wks.; basal diet 4 wks.; 3′-Me-DAB 2 wks.; basal diet at least 8 wks.	3/7	43	1/18	5
D	3′-Me-DAB 4 wks.; basal diet 4 wks.;‡ 3′-Me-DAB 5 wks.; basal diet at least 8 wks.	15/50	30	1/16	6
E	3′-Me-DAB 4 wks.; basal diet 8 wks.; 3′-Me-DAB 4 wks.; basal diet at least 8 wks.	7/12	58	0/4	0
F	3′-Me-DAB continuous 8 wks.; Basal at least 8 wks.	25/26	96	12/33	36

* Data from fourteen separate series by C. C. Clayton, I. Segre, W. L. Miller, Jr., and H. W. Rumsfeld, Jr.

† Dietary variations during the middle period included the addition of 30 mg. of α-tocopherol/rat/day, 18 per cent yeast substituted for casein, caloric restriction, vitamin additions, added methionine, and the additive effects of substances such as urethan, methylcholanthrene, 2-acetylaminofluorene, DAB, atabrine, and selenium.

‡ In this series the metabolic rate was altered during the middle period.

occurred in fifteen females. When this carcinogen was fed in an interrupted series (Table 2, groups 1–2), the incidence was 8/8 or 100 per cent in the males but only 2/10 or 20 per cent in the females. When 4′-F-DAB was fed continuously for 14 weeks, the tumor incidence in the males was 50 per cent, as compared to zero in the females (Table 2, groups 9 and 10). There did not seem to be any consistent correlation between food and dye consumption or weight changes and tumor incidence in any of these experiments. Generally, in the interrupted series the heavier males lost more weight than the females during the initial feeding of dye, but the losses during the second dye period were approximately the same for the two groups (Table 2).

The differences in tumor incidence between the sexes were most apparent when intermediate doses of carcinogen were given. They were not evident when the dose of carcinogen was sufficiently high for maximal tumor production in the females, e.g., in groups 7 and 8 which received 3′-Me-DAB continuously for 14 weeks; or when the dosage of carcinogen was inadequate for a substantial tumor incidence among the males (Table 2, groups 5 and 6). These latter groups, incidentally, illustrate a difference in carcinogenicity between 3′-Me-DAB and 4′-F-DAB. Miller *et al.* have observed the latter compound to be highly carcinogenic[4] (13). In the present study this has been confirmed, but with the relatively low doses of carcinogen employed, the 4′F derivative was consistently somewhat less carcinogenic than 3′-Me-DAB (Table 2, groups 1 versus 3, 2 versus 4, 7 versus 9, and 8 versus 10).

The development of liver tumors in castrated males and in ovariectomized females is summar-

[4] Price, J. M.; Harman, J. W.; Miller, E. C.; and Miller, J. A. Progressive Microscopic Alterations in the Livers of Rats Fed the Hepatic Carcinogens, 3′-Methyl-4-Dimethylaminoazobenzene and 4′-Fluoro-4-Dimethylaminoazobenzene (in preparation).

TABLE 2

CONTROLLED EXPERIMENTS ON THE DEVELOPMENT OF LIVER TUMORS IN MALE AND FEMALE RATS FED 3′-Me-DAB OR 4′-F-DAB

Group*	Sex	Av. initial wt. (gm.)	Av. wt. end 1st dye feeding (gm.)	Av. wt. start 2d dye feeding (gm.)	Av. wt. end of dye feeding (gm.)	Av. food consumption on dye (gm/rat/day)	Survival at end of dye feeding	No. of tumors	Neg. survivors	Per cent tumors
1	M	215	184	264	254	12.7	8/12	8	0	100
2	F	181	172	219	205	14.9	10/12	2	8	20
3	M	224	206	280	257	12.1	12/12	1	11	8
4	F	186	173	227	193	12.2	12/12	0	12	0
5	M	228			264	14.5	15/15	5	10	33
6	F	172			191	11.6	15/15	0	15	0
7	M	208			249	11.8	15/15	15	0	100
8	F	157			195	11.9	15/15	14	1	94
9	M	233			266	12.9	8/8	4	4	50
10	F	181			202	12.3	8/8	0	8	0

*Groups 1–2: 0.064 3′-Me-DAB 4 wks; basal diet 4 wks.; 0.064 3′-Me-DAB 5 wks.; basal diet 16 wks.
Groups 3–4: 0.064 4′-F-DAB 4 wks.; basal diet 4 wks.; 0.064 4′-F-DAB 5 wks.; basal diet 16 wks.
Groups 5–6: 0.040 3′-Me-DAB continuous 12 wks.; basal diet 16 wks.
Groups 7–8: 0.040 3′-Me-DAB continuous 14 wks.; basal diet 16 wks.
Groups 9–10: 0.040 4′-F-DAB continuous 14 wks.; basal diet 16 wks.

TABLE 3

THE EFFECT OF CASTRATION ON THE DEVELOPMENT OF LIVER TUMORS IN MALE AND FEMALE RATS FED 3′-METHYL-DAB

Group*	Sex	Av. initial wt. (gm.)	Av. wt. end 1st dye feeding (gm.)	Av. wt. start 2d dye feeding (gm.)	Av. wt. end of dye feeding (gm.)	Av. food consumption on dye (gm/rat/day)	Survival at end of dye feeding	No. of tumors	Neg. survivors	Per cent tumors
1	M	236	192	259	230	13.0	8/10	4	4	50
2	F	172	151	205	175	12.9	8/10	1	7	13
3	M castrate	218	200	260	233	9.5	13/15	5	8	38
4	F ovariectomized	220	187	242	208	8.9	10/15	0	10	0
5	M†	215	184	264	254	12.7	8/12	8	0	100
6	F†	181	172	219	205	14.9	10/12	2	8	20
7	M castrate	224	198	278	253	10.9	11/15	6	5	55
8	F ovariectomized	190	167	234	214	8.7	9/15	4	5	44

* Groups 1–4: 0.064 3′-Me-DAB 4 wks.; basal diet 4 wks.; 0.064 3′-Me-DAB 4 wks.; basal diet 16 wks.
Groups 5–8: 0.064 3′-Me-DAB 4 wks.; basal diet 4 wks.; 0.064 3′-Me-DAB 5 wks.; basal diet 16 wks.
† Groups 5 and 6 are identical with Groups 1 and 2, Table 2.

ized in Table 3. In the initial series (groups 1–4), the animals were not of comparable ages; but in the second series (groups 5–8), age, source, and past dietary history were carefully controlled. In both series castration tended to diminish the effect of sex on the formation of liver tumors due to 3′-Me-DAB, the effect being most evident in the more carefully controlled series in which castration decreased tumor incidence in the males from 100 to 55 per cent, whereas in the females castration increased tumor formation from 20 to 44 per cent. Again, these differences in tumor incidence did not seem to be due to differences in the consumption of food or of carcinogen.

Dye destruction in vitro.—The destruction of the azo dyes by liver slices or homogenates was essentially the same for tissues from male and female rats under most of the experimental conditions employed (Table 4). When the basal diet contained 10 μg. of riboflavin/gm, destruction reached 25 μg of DAB/100 mg of slices (wet)/hour, as compared to 13–14 μg DAB/100 mg/hour for rats fed 2 μg/gm of riboflavin (Table 4, groups 1–5). By way of contrast, the destruction of 3′-Me-DAB and 4′-F-DAB was consistently lower than that of DAB, ranging from 5 to 10 μg/100 mg/hour in these experiments. When 0.04 per cent of 3′-Me-DAB was fed for 2 weeks in the low riboflavin diet, destruction of the azo dyes by slices from the two sexes was also essentially similar (Table 4, group 6). However, when the feeding of dye was continued for 4 weeks, liver slices from males lost their capacity to destroy the three dyes more rapidly than slices from females (Table 4, group 7). The feeding of 0.04 per cent of 3′-Me-DAB also decreased the ability of fortified liver homogenates to destroy the dyes (Table 4, groups 10 and 11). The addition of flavin-adenine dinucleotide[5] (FAD) to these homogenates markedly increased the destruction of the dyes, as previously shown by Mueller and Miller (17), but did not bring it to the destruction rate of homogenates from rats fed high riboflavin. However, in the presence of added FAD both DAB and 3′-Me-DAB were destroyed at rates comparable to those obtained from rats fed the dye-free basal ration low in riboflavin (diet IV), while the destruction of 4′-F-DAB was increased to nearly twice that by livers from rats fed diet IV, viz., 11–13 μg/flask compared to 5–7 μg/flask (Table 4, groups 9–11). Dietary riboflavin appeared to be more important for the destruction of 4′-F-DAB and DAB by homogenates than for the destruction of 3′-Me-DAB (Table 4, groups 8 and 9).

TABLE 4

DESTRUCTION OF AZO DYES* BY SLICES OR HOMOGENATES OF RAT LIVER

GROUP	SEX	DIETARY SUPPLEMENT	TISSUE SYSTEM	AV. DYE DESTRUCTION† DAB	3′-Me-DAB	4′-F-DAB
				(μg/100 mg)		
1	M	10 μg B_2/gm for 2 wks.	Slice	18	6	9
	F	" "	"	19	7	8
2	M	" 4 wks.	"	20	8	9
	F	" "	"	21	9	9
3	M	" 6 wks.	"	25	9	11
	F	" "	"	25	9	10
4	M	2 μg B_2/gm for 2 wks.‡	"	14	7	6
	F	" "	"	14	6	6
5	M	" 4 wks.‡	"	13	6	5
	F	" "	"	13	6	5
6	M	0.04 per cent 3′-Me-DAB 2 wks.‡	"	10	5	5
	F	" "	"	10	6	5
7	M	" 4 wks.‡	"	4	1	1
	F	" "	"	11	5	5
				(μg/40 mg)		
8	M	10 μg B_2/gm for 6 wks.	Homogenate	43	16	32
9	M	2 μg B_2/gm for 4 wks.‡	"	15	7	7
	F	" "	"	12	7	5
10	M	0.04 per cent 3′-Me-DAB 4 wks.‡	"	1	0	1
			" plus FAD§	12	6	11
11	F	" "	"	4	0	0
			" plus FAD	16	5	13

* Values are averages for two rats, the value for each rat being an average of two flasks prepared from the same liver.

† Destruction values given as μg. dye/100 mg slices (wet)/hour. Destruction values given in μg. dye destroyed per flask by 0.4 cc. of 10 per cent homogenate per ½ hour.

‡ Groups 4–7 and 9–11 were fed 10 μg. B_2/gm for 2 weeks before receiving the diets listed.

§ 100 μg. of flavin-adenine dinucleotide added per flask.

DISCUSSION

The difference between the male and female rat liver in the response to 3′-Me-DAB is one of degree: livers of female rats fed this carcinogen

[5] Obtained from Mr. Philip Feigelson of this department.

developed tumors when the dose was sufficiently great or the time of feeding sufficiently prolonged. With lower doses, however, tumors developed less readily in the female than in the male, while castrates occupied an intermediate position. These unequal rates of tumor development apparently did not depend upon any inability of male liver to destroy the carcinogen; slices of livers from both sexes cleaved the dye at the same rate. Homogenates from the two sexes were likewise equivalent in their power to destroy dye. On the other hand, dye destruction *in vitro* varied with the level of riboflavin fed the rat, with the feeding of 3′-Me-DAB and with the addition of flavin-adenine dinucleotide to the reaction mixture. Differences were also marked among the rates at which any particular tissue destroyed the three dyes DAB, 3′-Me-DAB, and 4′-F-DAB. Thus, the test system employed appeared to be adequate for the detection of any major differences that might have existed between the ability of male and female liver to cleave these dyes. Since these livers appeared to be quite similar under all conditions imposed that did not involve a prolonged feeding of carcinogen, it was concluded that the different rates at which liver tumors developed in the two sexes probably depended upon some inherent factor other than those involved in dye destruction.

The prolonged feeding of carcinogen not only resulted in a greater visible damage (cirrhosis) to male than to female liver, but the ability to destroy dye reached a lower level in the male (Table 4). This latter impairment was interpreted to be a result of the greater sensitivity of the male liver to the dye rather than a cause of this sensitivity, although in a long-time experiment an induced impairment in ability to destroy dye would also tend to increase carcinogenesis.

The present results again illustrate biological differences between closely related carcinogens. No marked differences in tumor incidence have been reported between male and female rats fed the parent compound DAB. In a recent study[6] female rats were fed 0.06 per cent DAB for 16 weeks, and the incidence of tumors in the controls was 87 per cent, while the over-all incidence in the experimental groups was 48/56 or 86 per cent. This incidence is at least as high as that usually observed in male rats fed DAB (15, 18). By way of contrast, both 3′-Me-DAB and 4′-F-DAB are more carcinogenic in male rats than in females. However, the methyl derivative is not particularly sensitive to the amount of riboflavin in the diet (6–8), whereas 4′-F-DAB seems to be about as sensitive to the vitamin as DAB itself (13).

[6] H. W. Rumsfeld, Jr., unpublished data.

4′-F-DAB was included in this study at the suggestion of Dr. J. A. Miller as an example of a potent hepatic carcinogen in which a metabolic hydroxylation at the 4′-position would be unlikely. This latter reaction is known to take place in the metabolism of DAB (16, 21), and an analogous hydroxylation occurs at the para or 7-position in AAF (2), both yielding derivatives (3, 9, 12) that are either noncarcinogenic or only very weakly so. AAF is a much stronger hepatic carcinogen in male rats than in females, but this sensitivity to sex is considerably reduced when the 7-position is blocked with a fluorine atom.[7] By analogy, therefore, the carcinogenicity of 4′-F-DAB might have been independent of sex, but since male rats proved to be more sensitive to this carcinogen than females (Table 2), it would appear that the position para to the amine nitrogen does not have any particular significance in the unequal responses of the two sexes to these azo dyes.

Although no direct correlation is claimed between induced hepatomas in rats and spontaneous hepatomas in man, it is of interest that primary liver cancer in man is also more frequent in the male than in the female. This has been observed in many different series and is true not only in the United States, where the incidence of primary hepatoma is low, but also in Africa where primary carcinoma of the liver is the most common form of malignancy. The ratios between the number of men and women with liver tumors ranges from 3:1 to 6:1 (5, 11, 19). In addition, surveys among African natives indicate that men develop liver tumors relatively early in life.

SUMMARY

1. Male rats fed 3′-methyl-4-dimethylaminoazobenzene developed liver tumors more readily than females. This has been noted periodically over a 5-year period and has now also been demonstrated in controlled experiments. The incidence of induced liver tumors in castrates was intermediate between that in males and females.

2. 4′-Fluoro-4-dimethylaminoazobenzene also appeared to be more carcinogenic in male rats than in females. The sex differences in tumor incidence did not depend upon differences in the intake of food or of carcinogen.

3. The ability of liver slices or homogenates to destroy DAB, 3′-Me-DAB, or 4′-F-DAB *in vitro* did not vary with sex unless a carcinogen had been fed for a long period of time.

REFERENCES

1. Bielschowsky, F. Distant Tumours Produced by 2-Amino and 2-Acetyl-Aminofluorene. Brit. J. Exper. Path., 25:1–4, 1944.

[7] J. A. Miller and E. C. Miller, personal communication.

2. ———. A Metabolite of 2-Acetamidofluorene. Biochem. J., **39**:287–89, 1945.
3. ———. The Carcinogenic Action of 2-Acetyl-Aminofluorene and Related Compounds. Brit. M. Bull., **4**:382–85, 1946.
4. CLAYTON, C. C., and BAUMANN, C. A. Diet and Azo Dye Tumors: Effect of Diet during a Period When the Dye Is Not Fed. Cancer Research, **9**:575–82, 1949.
5. GELFAND, M. Malignancy in the African. South African M. J., **23**:1010–16, 1946.
6. GIESE, J. E.; CLAYTON, C. C.; MILLER, E. C.; and BAUMANN, C. A. The Effect of Certain Diets on Hepatic Tumor Formation Due to *m'*-Methyl-*p*-Dimethylaminoazobenzene and *o'*-Methyl-*p*-Dimethylaminoazobenzene. Cancer Research, **6**:679–84, 1946.
7. GRIFFIN, A. C., and BAUMANN, C. A. Hepatic Riboflavin and Tumor Formation in Rats Fed Azo Dyes in Various Diets. Cancer Research, **8**:279–84, 1948.
8. GRIFFIN, A. C.; CLAYTON, C. C.; and BAUMANN, C. A. The Effects of Casein and Methionine on the Retention of Hepatic Riboflavin and on the Development of Liver Tumors in Rats Fed Certain Azo Dyes. Cancer Research, **9**:82–87, 1949.
9. HOCH-LIGETI, C. Effect of Feeding 7-OH-2-Acetaminofluorene to Albino Rats. Brit. J. Cancer, **1**:391–96, 1947.
10. LEATHEM, J. H. Influence of Sex on 2-Acetylaminofluorene-induced Liver Tumors in Rats and Mice. Cancer Research, **11**:266, 1951.
11. LICHTMAN, S. S. Diseases of the Liver, pp. 717–48. 2d ed. Philadelphia: Lea & Febiger, 1949.
12. MILLER, J. A., and MILLER, E. C. The Carcinogenicity of Certain Derivatives of *p*-Dimethylaminoazobenzene in the Rat. J. Exper. Med., **87**:139–56, 1948.
13. MILLER, J. A.; SAPP, R. W.; and MILLER, E. C. The Carcinogenic Activities of Certain Halogen Derivatives of 4-Dimethylaminoazobenzene in the Rat. Cancer Research, **9**:652–60, 1949.
14. MILLER, W. L., JR., and BAUMANN, C. A. Basal Metabolic Rate and Liver Tumors Due to Azo Dyes. Cancer Research, **11**:634–39, 1951.
15. MINER, D. L.; MILLER, J. A.; BAUMANN, C. A.; and RUSCH, H. P. The Effect of Pyridoxin and Other B Vitamins on the Production of Liver Cancer with *p*-Dimethylaminoazobenzene. Cancer Research, **3**:296–302, 1943.
16. MUELLER, G. C., and MILLER, J. A. The Metabolism of 4-Dimethylaminoazobenzene by Rat Liver Homogenates. J. Biol. Chem., **176**:535–44, 1948.
17. ———. The Reductive Cleavage of 4-Dimethylaminoazobenzene by Rat Liver: Reactivation of Carbon Dioxide-Treated Homogenates by Riboflavin-Adenine Dinucleotide. *Ibid.* **185**:145–54, 1950.
18. RUSCH, H. P.; BAUMANN, C. A.; MILLER, J. A.; and KLINE, B. E. Experimental Liver Tumors. A.A.A.S. Res. Conf. on Cancer, pp. 267–87, Lancaster: Science Press, 1945.
19. SPATT, S. D., and GRAYZEL, D. M. Primary Carcinoma of the Liver. Am. J. Med., **5**:570–73, 1948.
20. STASNEY, J.; PASCHKIS, K. E.; CANTAROW, A.; and ROTHENBERG, M. S. Neoplasms in Rats with 2-Acetaminofluorene and Sex Hormones. II. Cancer Research, **7**:356–62, 1947.
21. STEVENSON, E. S.; DOBRINER, K.; and RHOADS, C. P. The Metabolism of Dimethylaminoazobenzene (Butter Yellow) in Rats. Cancer Research, **2**:160–67, 1942.

In Memoriam

George Milton Smith

1879–1951

"By their fruits ye shall know them." The death of George Milton Smith on February 26, 1951, is now history. To record the vital role he played in the story of this journal is singularly appropriate, for much of it now is only known to a very few of his close associates, and, also, because it typifies the quiet, persistent, and efficient approach that characterized so many of the contributions of this great crusader in the cause of cancer.

In 1916, when the *Journal of Cancer Research* was founded, Dr. Smith was the Director of the Barnard Free Skin and Cancer Hospital. Well versed in the best traditions of pathology as an associate of the renowned Eugene Opie and familiar with the pioneering studies of Leo Loeb, one of the original five editors, he was keenly sympathetic with the plan of the American Association for Cancer Research to confine this publication to results of broadening investigation then becoming evident. It would lead far afield to detail the tribulations of the journal and its various sponsors during the next quarter century and more. Now it may be said that Dr. Smith's devotion to the plan remained inviolate, but, more important, his influence attained such stature that reinstatement of the journal in the Association with its high purpose to further research, now so well met, is more largely due to George M. Smith than to the combined efforts of all others. His determination never faltered, his perseverance never tired, and when the opportunity to consummate the original objectives arose, he grasped it successfully to the great advantage of us all.

How such exemplary characteristics as George Smith combined are acquired can only be approached by recalling the exceptionally broad and varied experiences of his life. His parents were citizens of the United States; his father, a Dane by descent, was Master of Ships in the China Sea, and George was born in Hong Kong. At the age of 8, he was at school in Hamburg, Germany, and 2 years later in Boston, Massachusetts, where he remained until the Yale days that continued, with only interruptions for professional education and national service; and he became characterized increasingly by unswerving and intense loyalty with the progress of his distinguished life.

His professional career had a similarly broadly patterned foundation, concentrating gradually with preparation and opportunity in a few scientific problems to which he made signal contribution. School days in medicine at Columbia and clinical training during 4 more years, preceded 7 years in pathology at Washington University, climaxed, as has been said, by the directorship of the affiliated cancer hospital. World War I interrupted; George was a Captain in the Army Medical Corps of the Expeditionary Forces. And in World War II, he not only wisely directed the Connecticut State Defense and War Council, he was as well consultant to Army's Surgeon General and chairman of the National Research Council's Committee on Armored Vehicles. His pioneering and continuing experience in industrial medicine, as medical director of the Scoville Manufacturing Company from 1920, has proved of high importance nationally and also has promoted the organization of this discipline as an educational enterprise.

Many and large as these several services have been, they do not compare with the fruit of Dr. Smith's dominant medical absorption during his last 20 years. Concerned with problems of cancer early in his professional career, his was a major role in the expansion of the splendid effort now pointed toward understanding of this malady of many expressions, and of its prevention and treatment. Informed of the detail, acquainted with the investigators throughout the world, as medical director of the Anna Fuller Fund since its inception, advisor to the International Cancer Research Foundation, member of the Board of Scientific Advisers of the Jane Coffin Memorial Fund for Medical Research, member and later executive director of the National Advisory Cancer Council, the scope of relations and the responsibilities he bore so magnificently was indeed unrivaled.

It has been well said that the problems of cancer are not only of primary importance to medicine and the health of man but also that they involve the deep mysteries of organic growth and development, and these touch all fields of biological science. The campaign against cancer is now being waged on a scale so vast that it requires not only intensive research by a host of workers, but statesmanlike co-ordination of the activities and wise administration of the generous funds now available for its conduct. Fighters are needed in the front line, but a sound board of strategy and efficient service of supply is of equal importance. In providing these George M. Smith was particularly distinguished.

Obviously George had great abilities but none so large as his understanding of his fellow-men and his unswerving purpose to aid them in the varied fashions that his wide experience in science and in life had provided. He was tolerant of frailty and always at hand to help in the dilemmas that all too frequently arise; he was quick to recognize talent and to provide every possible support for its maturation. It may well be said, as the shadows lengthen and the years roll by, that his influence will be diffused, but even as it spreads it will grow in accomplishment.

M. C. Winternitz, M.D.